T FOLLICULAR REGULATORY CELLS PROMOTE THE GERMINAL CENTER REACTION AND ALLERGIC IGE RESPONSE WHILE REPRESSING ABNORMAL DIFFERENTIATION OF T FOLLICULAR HELPER CELLS

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DEDICATION

To my Friend and Savior - The Almighty Lord,

Who has built me who I am and put me where I need to

be. I can do nothing without You, my Lord.

“For I know the plans I have for you,” declares the LORD, “plans to prosper you and not to harm you, plans to give you hope and a future. Then you will call on me and come and pray to me, and I will listen to you. You will seek me and find me when you seek me with all your heart. I will be found by you.” -- Jeremiah 29: 11-14

ACKNOWLEDGEMENT

To pursue the dream of becoming a scientist is never easy, neither is the process of graduate school training. It took several villages of people who helped/supported me to survive till this stage finishing my PhD. I have endless gratitude to all the people I came across in the past five years, no matter you helped/supported or criticized/judged me. You all contributed to building up who I am now and who I will be in the future.

Overall, I greatly enjoyed my PhD training especially after joining the lab of Dr. Alex Dent. When I applied for PhD programs, I made up my mind to pursue biochemistry/cancer biology and would never want to touch on immunology. Much of my interest was kinase/signaling pathway regulations. However, many times things just don't go as we want or bet. I joined Alex's lab doing immunology research and after all these years' training, I have much less interest remain for kinase/signaling regulation, developed greater interests into cellular immunology (*in vivo*). Surely this will affect my future directions and career interests.

I never thought I would join Alex's lab four years ago. Unlike many of my cohort students who easily found a lab to join after three rotations, I did in total 5 rotations, largely because I was a "trouble maker". Three of the labs I rotated moved out of Indiana shortly and I had no choice by then, either quit the program or just had one last/extra rotation—the staff of the graduate division was worried about me and warned me that I should not ruin the last rotation. I did the last one

with Alex and survived joining the lab, still I had no idea what immunology research was by then. When I look back I always feel so grateful that Alex took and believed me. Nobody would argue with me that Alex is a super great mentor, I'm so blessed to be his student. He's like a father guiding me from a "toddler" to a "man" in the research of immunology. When I say "father" I mean the great kindness, the patience, the support, and love. Like in a family, of course, we had several occasions that we're mad at each other and lost temper, though I don't think that affected our bond. He's the one who I talked with nearly every day in the past years, who I could ask any kind of questions and discussed any scientific/life thoughts. Numerous times I turned to him for my highs or lows throughout my training, he always put aside his things in hands and encouraged me to thrive. The foremost thing I appreciate him is that he trained me to become a good immunologist and showed me how to be a good scientist. This is something that will have a great impact in my future career and maybe even whole life. I could accomplish very little without his mentorship in those years.

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My sincere regards to everyone who contributed to my growth throughout years. Thank you all.

Ming Xie

T FOLLICULAR REGULATORY CELLS PROMOTE THE GERMINAL CENTER REACTION AND ALLERGIC IGE RESPONSE WHILE REPRESSING ABNORMAL DIFFERENTIATION OF T FOLLICULAR HELPER CELLS

Follicular T helper (TFH) and regulatory (TFR) cells are two key classes of CD4+ T cells found in germinal centers (GCs). The primary role of TFH cells is to help B cells form GCs to produce high-affinity antibodies during an infection while the role of TFR cells remains controversial. The transcriptional repressor Bcl6 is essential for the differentiation of TFH, TFR and GCB cells and understanding signaling pathways that induce Bcl6 and TFH cell differentiation are important. We observed that Bcl6 is highly up-regulated in activated CD4 T cells following glucose deprivation by a pathway involving the metabolic sensor AMP kinase. The transcription factor Blimp1 represses both TFH cell differentiation and Bcl6 expression, and we show the major role of Blimp1 on TFH cell differentiation is to repress Bcl6 expression and not other genes in the TFH differentiation pathway. We also found Bcl6 positively regulates expression of the key TFH cell receptor PD-1 by inhibiting the repression of PD-1 by the transcription factor Tbet. The roles of TFH and TFR cells in controlling allergen-specific IgE were investigated using a peanut allergy model and strains of mice with alterations in the TFH and TFR pathways. We found TFR cells unexpectedly play an essential role in promoting and maintaining IgE production and anaphylaxis, as well as the GC reaction. Compared to control mice, TFR-deficient mice lacked circulating peanut-specific

IgE and anaphylactic responses were significantly weakened. Mechanistically, TFR cells require Blimp1 controlled IL-10 to promote GCB cell survival and IgE production. Blocking IL-10 signals mimicked the loss of IgE levels in TFR-deficient mice and rescued mice from anaphylaxis. Overall, these studies have defined novel roles of Bcl6, TFH and TFR cells in regulating antibody production by the GC reaction, and provide greater understanding of how allergic immune responses are controlled.

Alexander L. Dent, Ph.D., Chair

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2DG	2-Deoxy-D-glucose
Ab	Antibody
ADP	Adenosine diphosphate
Ag	Antigen
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AMP	Adenosine monophosphate
AMPK	Adenosine Monophosphate-activated Protein Kinase
APC	Antigen presenting cell
Bcl6	B cell lymphoma 6
Bcl6FC	*Bcl6*$^{fl/fl}$Foxp3cre mice
BCR	B cell receptor
Blimp1	B lymphocyte-induced maturation protein-1
Blimp1FC	*Blimp1*$^{fl/fl}$Foxp3cre mice
BoyJ	B6.SJL-PrprcaPepcb/BoyJ
BSA	Bovine serum albumin
CD40L	CD40 ligand
CHIP	Chromatin immunoprecipitation
cKO	Conditional knockout mice
CTBP	C-terminal-binding protein
cTFH	Circulating T Follicular Helper
cTFR	Circulating T Follicular Regulatory
CTL	Cytotoxic T lymphocyte

CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCR5	C-X-C motif receptor 5
D36	Day 36
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DTR	Diphtheria toxin receptor
dcKO	*Bcl6*$^{fl/fl}$*Blimp1*$^{fl/fl}$Cd4cre mice
DEG	Differentially Expressed Genes
DKO	*Bcl6*$^{fl/fl}$*Blimp1*$^{fl/fl}$Foxp3cre mice
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EOMES	Eomesodermin
FACS	Fluorescence-activated cell sorting
Fas	TNF receptor superfamily member 6
FBS	Fetal Bovine Serum
FDC	Follicular dendritic cell
Fgl2	Fibrinogen-like protein 2
Fig.	Figure
GATA3	GATA binding protein 3
GC	Germinal center
GD	Glucose deprivation
GITR	Glucocorticoid-induced TNFR-related protein
GZMB	Granzyme B

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
ICOS	Inducible T-cell costimulatory
ICOSL	Inducible T-cell costimulatory ligand
ICS	Intracellular staining
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
i.g.	Intragastric
IL	Interleukin
IL2R	Interleukin-2 receptor
IL21R	Interleukin-21 receptor
i.p.	Intraperitoneal
IRF4	Interferon regulatory factor 4
KLH	Keyhole limpet hemocyanin
KO	Knockout mice
MDS	Multidimensional scaling
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
mLN	Mesenteric lymph node
MTOB	4-Methylthio-2-oxobutanoic acid
mTorc1	Mammalian target of rapamycin complex 1
NFAT	Nuclear factor of activated T-cells
NP	4-hydroxy-3-nitrophenyl acetyl

OVA	Ovalbumin
PARP1	Poly (ADP-Ribose) Polymerase 1
PBMC	Peripheral Blood Mononuclear Cell
PCT	Peanut and Cholera Toxin
PD-1	Programmed cell death protein 1
pMHCI	MHC I-peptide complex
pMHCII	MHC II-peptide complex
Prf1	Perforin1
PtenFC	*Pten*$^{fl/fl}$Foxp3cre mice
pTreg	Peripheral T regulatory
QPCR	Quantitative RT-PCR
RNAseq	RNA sequencing
RORα	Retinoic acid receptor-related orphan receptors alpha
RORγ	Retinoic acid receptor-related orphan receptors gamma
RV	Retrovirus
SLAM	Signaling lymphocyte activation molecule
SIV	Simian Immunodeficiency Virus
SLE	Systemic Lupus Erythematosus
SRBC	Sheep red blood cell
STAT	Signal transducer and activator of transcription
Tbet	T-box transcription factor TBX21
Tcf7	Transcription Factor 7
TCR	T cell receptor

TFH	T Follicular Helper
TFR	T Follicular Regulatory
TGF-β	Transforming growth factor beta
Th	T helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory
TSLP	Thymic stromal lymphopoietin
tTreg	Thymus T regulatory
WT	Wild Type

INTRODUCTION

Innate and adaptive immunity

The immune system defends the body against infectious pathogens and pathological tumor growth while uncontrolled immune responses could cause allergic or autoimmune diseases (1). Innate immunity and adaptive immunity are two major arms of the immune response that work cooperatively to generate effective protection against pathogens following infection or vaccination (1-3).

Innate immunity

The first line of defense is the innate immunity with mechanisms in place ready for quick response to infections. Outer anatomical barriers like skin and mucosal surfaces are the first innate defense protecting us from infectious pathogens such as virus and bacteria (4, 5). Innate immune cells derived mostly from common myeloid progenitors form additional innate defenses in addition to the physical barriers mentioned above. The major types of innate immune cells include dendritic cells (DCs), macrophages, monocytes, mast cells, basophils, neutrophils, eosinophils and natural killer (NK) cells. Innate immune cells express pattern recognition receptors (PRRs) either on their surface or in the cytoplasm. One major type of PRR is toll-like receptors (TLRs) which recognize pathogen associated molecular patterns (PAMPs) such as double stranded RNA and mannans. This recognition of PAMPs by PRRs leads to phagocytosis and killing of pathogens (3, 6).

Macrophages and DCs differentiate from monocytes in the bone marrow and they are professional antigen presenting cells (APCs) (3, 7, 8). The differentiation could be triggered by different environmental cues. Once matured, they circulate into peripheral blood, spleen or lymph nodes (LNs) and present ingested antigens (Ags) to T or B cells as the link between innate and adaptive immunity.

Mast cells, neutrophils, eosinophils and basophils are granulocytes which all have granules in cytoplasm (7-13). However, each type of granulocytes contains distinct granules that function differently. Mast cells and basophils share similarities of function and morphology. Both mast cells and basophils have granules which contain chemical mediators like histamine, leukotrienes and heparin (11, 12). Activation through binding/crosslinking the surface receptors of immunoglobulin E (IgE) triggers the degranulation of mast cells and basophils and the release of chemical mediators cause atopic diseases. Thus, mast cells and basophils are two major types of innate immune cells involved in type 2 immunity (12). Chemical gradients of molecules like interleukin-8 (IL-8) and leukotrienes generated at infection sites are signals for neutrophils to phagocytose opsonized microbes or particles. On the other hand, neutrophils release cytokines and antimicrobial contents by degranulation to recruit additional immune cells to amplify the inflammatory responses (6). Eosinophils release cytokines and enzymes such as peroxidase, ribonuclease, prostaglandins and neurotoxin upon activation by factors like IL-5. Through the degranulation and release of cytokines

and enzymes, eosinophils facilitate killing of infected cells or pathogens (10). However, degranulation of eosinophils could cause problems during autoimmune or/and allergic diseases.

Adaptive immunity

APCs are the fundamental link between innate and adaptive immunity (1-3). Naïve T cells first develop in the thymus and the main types are CD4+ or CD8+ T cells. Later they circulate into the peripheral sites such as blood or lymph nodes (LNs). APCs express class II MHC molecules which present antigenic peptides which will be recognized by T cell receptor (TCR) to activate CD4+ T cells and this process typically happens near the site of infection. Class I MHC is expressed by all nucleated cells and presents peptide Ags to CD8+ T cells. Development of B cells primarily happens in the bone marrow and they do not need MHC for activation or selection. There are two major classes of B cells: B1 and B2 cells (14). Marginal zone and follicular B cells are the two subclasses of B2 cells. B1 progenitors in fetal liver give rise to B1 cells which are T cell independent, for they do not need help from T cells to produce antibodies (14). Antibodies produced by B1 cells are polyspecific and can bind to self or microbial Ags. B cell progenitors in the bone marrow differentiate into transitional B cells and later develop into B2 cells. B2 cells give rise to either marginal zone or follicular lineages (14). Both T and B cells undergo positive and negative selection through different mechanisms to make sure they distinguish between self and non-self Ags. This is a critical

process to avoid autoreactive cell development and to create diverse T and B cell repertoires which could respond to different foreign Ags.

CD8+ T cells are usually called cytotoxic T lymphocytes (CTLs) as they release granzyme and perforin containing granules to kill infected cells or tumor cells (15). Another subset of T cells are Natural killer T cells (NKT) which express NK receptors and invariant TCR. NKT cells are also important for adaptive immune responses. NKT cells produce cytokines such as IL-4, IL-9 and IFNγ and their roles have been identified in immune defense like viral infection and tumor immunity (16-20).

A major function of the adaptive immune response is to produce highly specific antibodies (Abs) that bind to antigen (Ag) with high affinity and help eliminate pathogens and foreign substances.

B cells which produce Abs for humoral immunity could produce five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM. There are also several subtypes of IgA and IgG. Abs can mediate protection against pathogens by various means: 1) Ab-dependent cell-mediated cytotoxicity; 2) Ab triggered signal transduction leading to receptor activation; 3) Ab-mediated neutralization of free Ags; 4) Ab-mediated complement opsonization; 5) phagocytosis of opsonized pathogens. Ab isotype switch and long-lived plasma cell differentiation typically occurs within germinal centers (GCs), specialized immune responses located in

peripheral LNs and other secondary lymphoid organs. Several cell types contribute to the GC responses including follicular helper T, follicular regulatory T and follicular DCs. GC reactions of these cell types will be discussed in more detail below.

CD4+ helper T cell subsets

T helper cells (Th), or CD4+ T cells, is one major type of T cells which play essential roles in adaptive immune system. Their primary job is to provide help to other immune cells such as APCs and B cells through production of cytokines. The differentiation of different subsets of CD4+ T cells require several factors: 1) T cell receptor (TCR) signal from APCs through the presentation of pathogens by MHCII; 2) costimulatory signals mediated by cell surface interactions; 3) cytokine environment which could trigger the signal transducer and activator of transcription (STAT), NF-κB or SMADs signaling pathways. These cytokine-specific activated signaling pathways further induce downstream transcription factors of each specific cell lineages. Each of the Th cells produce their own signature cytokines that providing either help or suppression to other immune cells. As showed in Fig. 1, the major CD4+ subsets include but not limited to type 1 T helper (Th1), Th2, Th9, Th17, T follicular helper (TFH) and T regulatory (Treg) cells (21). Each type of Th cells has their own master transcription factors, requirements of differentiation, signature cytokine production and functions.

Th1 cells are generated during intracellular infection by pathogens such as viruses or bacteria. APC produced IL-12 binds to the receptor to activate STAT4 signaling which triggers the expression of the Th1 master transcription factor T-box 21 (*TBX21* or *Tbet*) (22). STAT4 together with Tbet promotes the differentiation of Th1 cells from naïve CD4+ T cells (23). The signature cytokine produced by Th1 cells is IFN-γ which could stimulate innate immune cells like macrophages to clear invading pathogens.

It is also significant that IFN-γ produced by Th1 cells further stimulates STAT1 pathway to strengthen Tbet expression (24). Th1 response is critical for pathogenic infection clearance while uncontrolled type 1 response leads to chronic inflammation or autoimmune diseases.

Th2 cells provide protection against extracellular parasites and *Gata3* is the master transcription factor for Th2 cells (25). IL-4 activates the STAT6 pathway and subsequently induces GATA3 expression (26). IL-2 and thymic stromal lymphopoietin (TSLP) are the two other important cytokines mediating Th2 differentiation through STAT5. Th2 cells produce cytokines IL-4, IL-5 and IL-13 which involve in atopic diseases. IL-4 promotes B cell production of IgE, the primary mediator of allergic diseases (27).

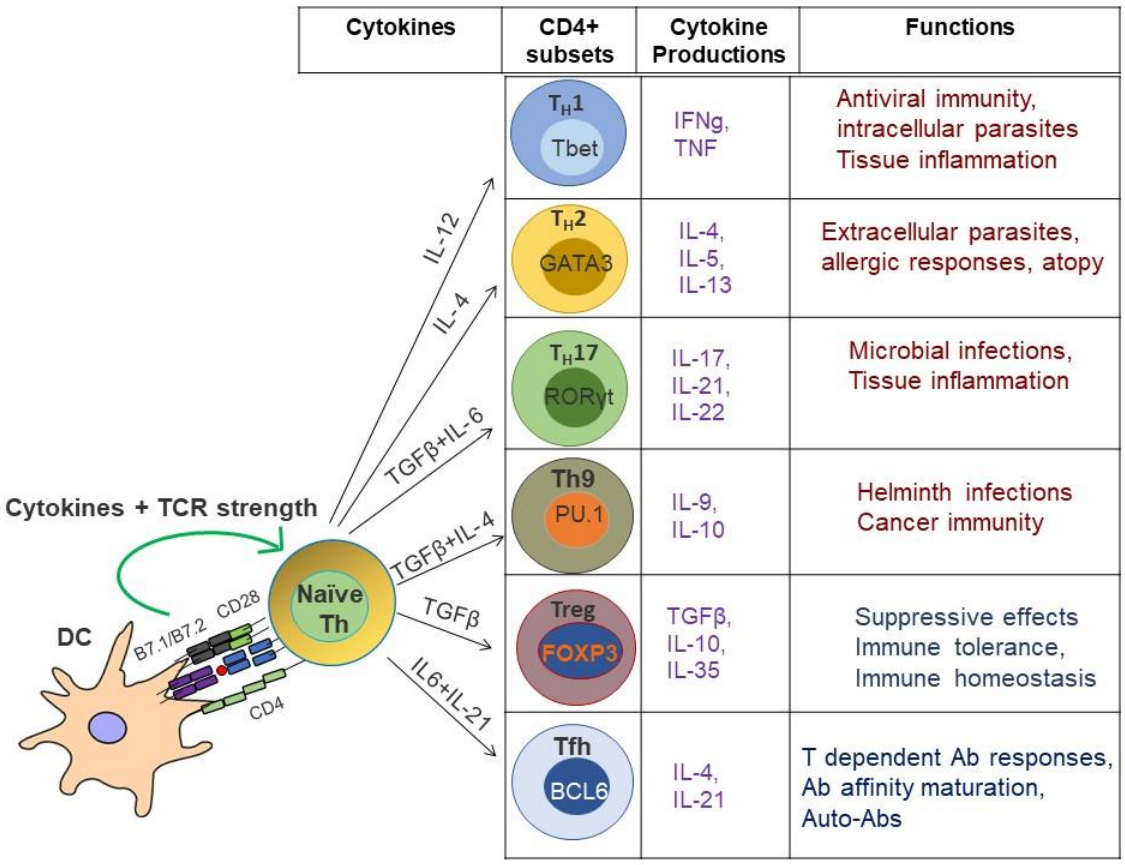

Figure 1: The differentiation of CD4+ T cell subsets and their functions.

Similar to Th2 cells, Th9 cells mainly help eliminate extracellular pathogens such as helminths and parasites (28). Th9 cells also play critical roles in the development of allergic diseases like asthma (29). Transforming growth factor beta (TGFβ) together with IL-4 activate PU.1 and interferon regulatory factor 4 (IRF4) to promote the differentiation of Th9 cells (30). The major cytokine produced by Th9 is IL-9. IL-9 is a growth factor for mast cells and promotes IgE production by B cells (31, 32). Recent studies show that Th9 cells play critical roles in promoting antitumor immune responses (33-35).

Naïve CD4+ T cells differentiate into Th17 cells under the cytokine environment of IL-6, TGFβ and/or IL-23 (36). Cytokine activated STAT3 upregulates the master regulator of Th17- retinoic acid receptor-related orphan receptor gamma t (RORγt) (37). Th17 cells produce IL-17 for the protection against fungi or bacteria and they are mainly located at mucosal surfaces. Th17 cells are the primary pathogenic immune cells in multiple autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, lupus and psoriasis (38, 39).

Foxp3+ Treg cells are the fundamental suppressive cells that control immune tolerance (40, 41). Treg cells develop either the thymus or peripheral sites and the origins separate Treg into thymic Treg (tTreg) and peripheral Treg (pTreg) (42). The differentiation of pTreg requires TGFβ and IL-2 while nTreg development is more complex. Treg cells need IL-2 for their development and they express high level of the IL-2 receptor CD25. Foxp3 is the master transcription factor of all Treg

cells and is located on the X chromosome (43). The main function of Treg cells is to maintain immune tolerance through the production of IL-10, TGFβ and other suppressive cytokines (44). Deficiency of Treg or Foxp3 causes severe autoimmune and inflammatory diseases (45, 46). However, excessive Treg cells prevent the immune system from eliminating pathogens or expanding tumor cells (47, 48). Several studies have shown that TFR cells primarily differentiate from Foxp3+ Treg precursor cells (49-53), however, like TFH cells, TFR cells can also develop from naïve CD4 T cells (54). Tregs are generated either during T cell differentiation in the thymus (tTregs) or from mature CD4 T cells in the periphery (pTregs) (55, 56), but whether TFR cells preferentially develop from tTregs or pTregs is not known. TFR cells are discussed below in much more detail.

Naïve CD4+ T cells can also differentiate into follicular helper T cells (TFH) under the cytokine influence of IL-6 and IL-21. TFH cells are mainly located in germinal center and are a pivotal cell type that regulates B cell responses in the GC as discussed below in more detail.

Germinal center reaction

The GC is a cluster of rapidly proliferating and clonally expanding B cells where they undergo somatic hypermutation of immunoglobulin (Ig) genes, Ig affinity selection, and class switch recombination (Fig. 2 and Fig. 3) (57, 58). Long-lived memory B cells and plasma cells are generated within GCs to produce high-affinity Abs against pathogens. The master transcription factor for TFH cells, *Bcl6*,

is also highly expressed in GC B cells (59). Unique cell surface markers of GC B cells are GL7 and Fas (TNF receptor superfamily member 6) in additional to other markers such as IL21R, ICOSL and SLAM (60). Extensive studies have shown GC B cells closely interact with TFH cells to receive their help. Signals gathered from B cell Ag presentation to T cells such as CD40 and its ligand CD40L are essential for the development of GC B cells (61-64). It is well accepted that TFH cells are the primary source of CD40 signals within the GCs. Upon the recognition of cognate Ag, B and T cells are activated in the primary follicle and T cell zone, respectively (65). B and T cells interact with each other after their migration to the interfollicular region. Later, both B and T cells migrate into the follicle, the migration of B cells need the network with follicular DCs. Proliferating B cells within the follicle result in the formation of the early GC and it continues its expansion which leads to the establishment of the mature GC and typically this process takes about 7 days (65).

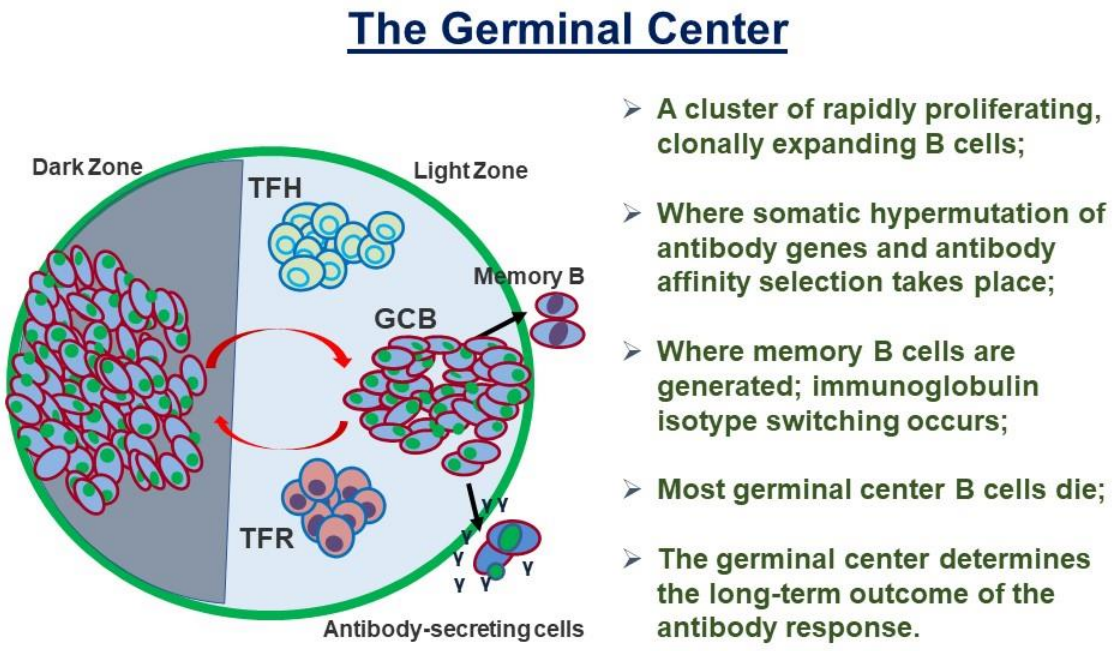

Figure 2: Germinal center distribution and functions. Ab isotype switch and long-lived plasma cell differentiation typically occurs within GCs, specialized immune responses located in peripheral LNs and other secondary lymphoid organs. Several cell types contribute to the GC responses including TFH, TFR and follicular DCs. The GC is a cluster of rapidly proliferating, clonally expanding B cells where somatic hypermutation of Ab genes and Ab affinity selection takes place. Most GC B cells die and the GC determines the long-term outcome of the high affinity Ab response.

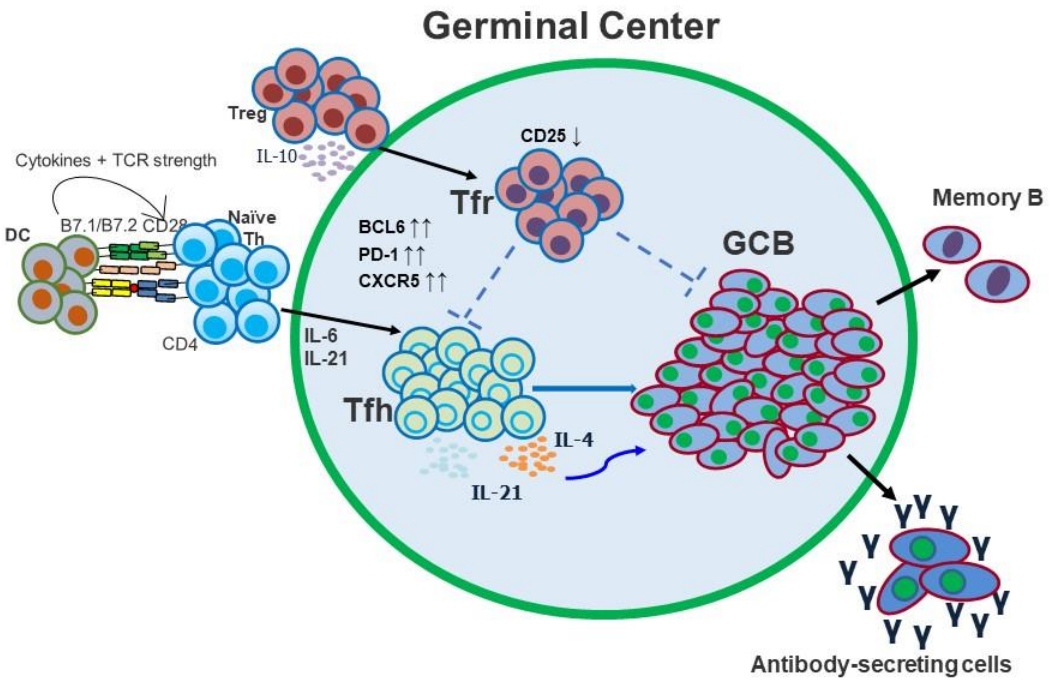

Figure 3: TFH and TFR cells both act in the germinal center (GC) to regulate the generation of antigen-specific antibody-secreting cells. Historically, TFR cells were considered to only suppress TFH and GC B cells, however, work in this thesis alters that idea. TFH cells differentiate from conventional CD4 T cells after activation with Ag and DC presentation. TFR cells differentiate from conventional Treg cells and migrate into the GC.

There are two highly organized zones within the GCs: the dark zone and light zone (65). B cells located in the dark zone are called centroblast while light zone B cells are centrocytes (66). TFH and follicular DCs provide positive signals to centrocytes for selection in the light zone and positively selected GC B cells cycle into dark zone to proliferate and acquire mutations, after which centroblast cycle back to light zone for selection again. Several cytokines have been reported as key regulators of GC zones. IL-21, produced primarily by TFH cells in the GCs, plays essential roles in instructing the cycle transition from centrocyte to centroblast while another TFH-produced cytokine, IL-4, is required for the acquisition of the centrocyte state (67). Recently, Craft and colleagues showed that the regulatory cytokine IL-10, which is produced by TFR cells is also important for the development of GC B cells. Specifically loss of TFR-derived IL-10 directly caused the loss of dark zone (68).

T follicular helper cells

A specialized subset of differentiated CD4+ T cells, TFH cells, are required in the germinal center reaction to help B cells generate high affinity Abs to Ag (69, 70). TFH cells control the initiation as well as the outcome of the GC B cell response (71-74). TFH cells are critical for the proper production of protective Abs during an infection, however, the over-production of TFH cells can also lead to autoimmunity since TFH cells can help B cells to produce self-reactive Abs (74-76). Thus, the proper regulation of TFH cell differentiation is essential both for normal immune function and for preventing autoimmune disease.

During an immune response, Ag-activated CD4 T helper cells that up-regulate the expression of the transcription factor Bcl6 and down-regulate the transcription factor *Blimp1* develop into TFH cells. The function of TFH cells is to promote formation of GCs and select B cell clones that produce high-affinity Abs (reviewed in (51, 69-71, 77)). TFH cells are commonly identified as CD4+ CXCR5+ and PD-1+ T cells. TFH cells have an activated effector T cell phenotype but do not express the high affinity IL-2 receptor (IL2R, also known as CD25). TFH cells control the outcome of the GC B cell response, and are critical for memory B cell and plasma cell development. TFH cells produce IL-21, a cytokine that potently promotes B cell activation and Ab secretion. While TFH cells are required for the production of high affinity Abs, excessive numbers of TFH cells can promote autoimmunity by helping B cells produce self-reactive Abs (74-76). The proper regulation of TFH cell differentiation is therefore essential for strong ab responses and preventing development of autoimmune disease (75, 76).

The transcription repressor BCL6 is a 95-kDa nuclear phosphoprotein (78). It contains a POZ/BTB domain at the N-terminus, three PEST domains in the central region and six Zinc Finger motifs by its C-terminus (78, 79). The POZ/BTB domain recruits several co-repressors while Zinc Finger motifs bind to specific promoter DNA sequences of target genes to repress transcription (78). Bcl6 is the master transcription factor of TFH cells, as overexpression of BCL6 favors differentiation of CD4 T cells into TFH cells. TFH cell differentiation is completely blocked in *Bcl6* conditional deleted T cells while other effector helper T cells are

relatively unaffected (80-82). It remains largely unknown why a naïve CD4 T cell develops into a TFH cell instead of other helper cell types (TH1, TH2, TH17) when it is activated in vivo. How the master transcription factor Bcl6 is induced during TFH cell differentiation also need to be fully elucidated. We wondered if metabolism controlled Bcl6 expression and TFH differentiation as previous reports showed that mature TFH cells have a unique limited metabolic activity compared to other effector T helper cells (83).

The stable activation of Bcl6 expression in CD4+ T cells during TFH cell differentiation has to overcome two critical impediments: 1) Bcl6 strongly auto-represses its own expression (84, 85), and 2) IL-2 produced during T cell activation inhibits Bcl6 expression (86-90). BCL6 auto-represses itself and this requires the co-repressor CTBP (C-terminal-binding protein) which associates with BCL6 in the Bcl6 promoter region (84). CTBP is regulated by NAD+ and NADH levels, which means it could be affected by metabolic stress (91, 92). Thus lowering NADH levels under metabolic stress potentially removes CTBP from binding with Bcl6 which reduces Bcl6 auto-repression. TFH-like cells can be produced *in vitro* with human CD4 T cells and STAT3/STAT4-activating cytokines plus TGFβ (93). However, these same signals do not promote mouse TFH cell differentiation, and in particular TGFβ plays different roles in mouse versus human CD4 T cell differentiation (93). Recently, Cyster and colleagues found that during an immune response, DCs can up-regulate expression of the IL2R in order to inhibit IL-2 signaling by CD4+ T cells and thus promote TFH cell differentiation (94). While this

DC-IL2R mechanism appears to be important for early TFH cell differentiation, more work is needed to understand the relevance of this pathway in different immune settings. Another mechanism for blocking IL-2 *in vivo* to promote TFH cell differentiation involves IL2R-expressing Treg cells (95). The significance of this pathway in different immune settings is also unclear. Another potential pathway for controlling IL-2 during the T cell response is through glycolysis, since decreased glycolysis leads to decreased cytokine translation (96).

Despite a great deal of investigation into Bcl6 function in CD4+ T cells, the precise mechanisms by which Bcl6 promotes TFH cell differentiation have not been fully elucidated. One major mechanism proposed for Bcl6 in the control of TFH cell differentiation is that Bcl6 inhibits terminal CD4+ T cell differentiation by repressing the *Prdm1* gene that encodes the transcription repressor Blimp1 (51, 70, 82, 87, 97). Blimp1 is a potent inhibitor of TFH cell differentiation, and IL-2 suppresses TFH cell differentiation largely through induction of Blimp1 expression (51, 87, 89). However, the mechanism by which Blimp1 itself represses TFH cell differentiation is not settled. Bcl6 is a direct target of Blimp1, and Bcl6 expression is repressed by Blimp1 (82, 98, 99). Thus, Blimp1 may inhibit TFH cell differentiation by simply repressing Bcl6 expression. At the same time, Blimp1 may repress other genes apart from Bcl6 that are critical for the TFH cell fate or TFH cell differentiation. Additionally, it has been proposed that Blimp1 promotes terminal T cell differentiation, which may indirectly promote differentiation away from a less differentiated TFH cell state (51, 70). Adding to the complexity of this

pathway is that Blimp1 represses Bcl6, which promotes TFH cell differentiation, but also represses genes that inhibit TFH cell differentiation, such as *Il2*, *Ifng* and *Tbx21* (Tbet) (98, 100). Thus Blimp1 can potentially either promote or inhibit TFH cell differentiation.

Although it is generally accepted that Bcl6 promotes TFH cell differentiation by repressing certain key genes (101, 102), the complete set of TFH cell differentiation genes regulated by Bcl6 have not been fully characterized and the overall process is not well understood. Since Bcl6 is a dedicated transcriptional repressor, genes that are turned on in TFH cells such as CXCR5, IL-21 and PD-1 must be regulated indirectly by Bcl6. In principle then, Blimp1 may repress the key genes required for TFH cell differentiation, and the critical function of Bcl6 in TFH cell differentiation may be to repress the anti-TFH cell activity of Blimp1. A hybrid model is that Blimp1 and Bcl6 each control different aspects of TFH cell differentiation, so that neither factor fully controls TFH cell differentiation independent of the other.

Mice deficient in Bcl6 or Blimp1 have not provided a clear answer as to the relationship between of Blimp1 and Bcl6 in the development of TFH cells (82, 103). Using conditional knockout (cKO) mouse models, researchers have shown that loss of Bcl6 in T cells results in complete loss of TFH cell development, while, conversely, Blimp1 cKO mice have increased TFH cell populations.

One important gene that can be used to assess Bcl6 and Blimp1 regulation patterns is *Pdcd1*, which encodes PD-1, a common marker for TFH cells. Bcl6 is known to promote PD-1 expression (104), and Blimp1 is a strong repressor of PD-1 (105). Since Bcl6 is a transcriptional repressor, the mechanism that Bcl6 uses to up-regulate PD-1 expression is likely to be an indirect pathway, such as by repressing the transcription of a gene which normally represses *Pdcd1* transcription. Therefore, one possibility is that Bcl6 promotes PD-1 expression primarily by repressing Blimp1-mediated transcriptional repression of *Pdcd1*.

T follicular regulatory cells

GC B cell responses are also regulated by T follicular regulatory (TFR) cells, which developed from Treg cells and localized to the GC (49, 50, 106-111)(Fig. 2 and 3). TFR cells are generally thought to limit the function of TFH cells in the GC (49, 50, 106-108, 111). TFR cells, like TFH cells, are dependent upon the transcriptional repressor protein Bcl6 for their development, but unlike TFH cells express the canonical Treg master regulatory transcription factor Foxp3 (49, 50, 106-111). The overall function of TFR cells is poorly understood, however the prevailing model for TFR cell function currently in the field is that TFR cells repress excessive TFH and GC B cell proliferation and promote the selection of high affinity B cells (49, 50, 106-108, 111).

Differentiation and regulation of TFR cells

Several studies have shown that TFR cells primarily differentiate from Foxp3+ Treg precursor cells (49-53)(Fig. 4), however, like TFH cells, TFR cells can also develop from naïve CD4 T cells (54). Tregs are generated either during T cell differentiation in the thymus (tTregs) or from mature CD4 T cells in the periphery (pTregs)(55, 56), but whether TFR cells preferentially develop from tTregs or pTregs is not known. Tregs in the intestinal mucosa are predominantly pTregs that develop in response to Ags derived from microbiota and diet as a tolerance mechanism (55, 56). TFR cells that develop in the gut lymphoid tissues such as Peyer's patches may therefore differentiate from pTregs, and so ultimately may have a naïve CD4+ T cell origin. Interestingly, Peyer's patch TFR cells have a markedly different transcriptome than peripheral lymph node TFR cells, possibly suggesting a different origin (112).

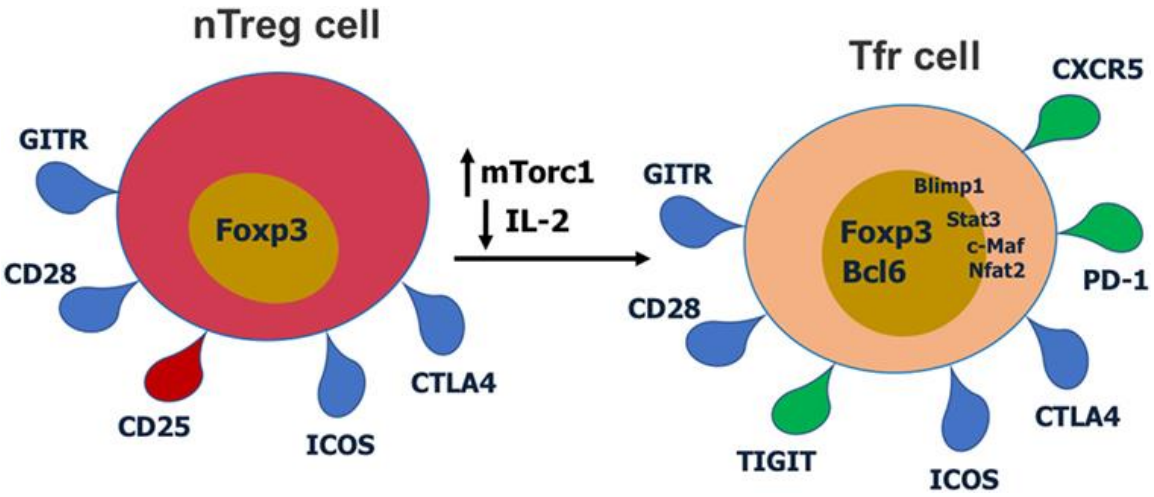

Figure 4: Cell surface receptors and transcription factors involved in TFR cells differentiation and function. nTreg cells differentiate into TFR cells when mTorc1 signal is increased while IL-2 signal is decreased. Blue colored proteins are known cell surface marker expressed on Tregs or TFR cells; green colored proteins are unique surface markers expressed by TFR cells; red colored CD25 is highly expressed on the surface of non-TFR Tregs but not TFR cells.

TFR cells express TFH cell surface markers such as PD-1, CXCR5 and ICOS, Treg surface markers such as CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and GITR (glucocorticoid-induced TNFR-related protein) and the master regulatory transcription factors for both Tregs (Foxp3) and TFH cells (Bcl6) (49, 50, 106, 109, 113). Thus, TFR cells display a hybrid or mixed TFH/Treg phenotype. Most studies have analyzed TFR cells in the mouse, but phenotypically similar TFR cells have also been described for humans (114, 115) and macaques (116). TFR cells also express significant levels of Blimp1 (49, 117). Notably, TFR cells express lower CD25 (the expression of CD25 on TFR cells is only about 50% of the expression of total Tregs) compared with non-TFR Tregs (109, 115). Together with Bcl6, Nfat2 (nuclear factor of activated T-cells 2) upregulates CXCR5 expression on Treg cells and enables them to migrate to GC, take on the follicular phenotype and become TFR cells (49, 50, 106, 109, 118). Recent work showed that mTorc1 is essential in regulating the conversion of Treg to TFR cells and this is potentially through a Stat3-Tcf-1-Bcl6 pathway (119, 120). Our lab has also specifically found that in contrast to TFH cells which can develop in the absence of Stat3, Stat3 is essential for TFR cell development (110).

Ag exposure triggers the differentiation of TFR cells and this process is DC-dependent (49, 50, 54, 113). Sage *et al*. used mice that express diphtheria toxin receptor (DTR) specifically on DCs to test this (107). DC-depletion led to substantially decreased TFR cells, however it is unknown which specific DC subsets directly contribute to TFR cell differentiation. At the same time, PD-1-

ligand expressed on DCs has an inhibitory role on TFR cell development (121). Treg cells can repress the function of APCs including DCs (122), but whether TFR cells can affect DCs or other APCs and how this might affect the GC response is unknown. Precisely what Ags and signals that Tregs respond to in order to become TFR cells is not well understood. TFR cells respond more strongly to self-Ags than foreign Ags, which fits with the self-reactive nature of tTregs (54, 123). While TFR cells can develop to have specificity for the immunizing Ag (54), a recent study on the TCR specificity of TFH and TFR cells indicated that in contrast to TFH cells, TFR cells do not proliferate well to the cognate Ag after immunization (53). Furthermore, an analysis of TCR gene sequences in TFH and TFR cells indicated that TFH cells are a sub-population of cells related to naïve CD4+ T cells, whereas TFR cells showed a TCR profile very similar to the total Treg cell population (53). These findings are consistent with a model that TFH cells are Ag-specific T cells that proliferate after Ag stimulation, while TFR cells develop in a polyclonal and Ag-independent manner from Tregs. Therefore, TFR cells either develop from Tregs in a polyclonal TCR-dependent response involving recognition of self-Ag, or TFR cells expand and differentiate through a Ag-independent and TCR independent pathway (e.g. Jagged1 plus Ox40 stimulation (124)). Note that the Maceiras *et al* (53) analyzed TFR cells from peripheral LNs, and the TCR specificity of Peyer's patch TFR cells may be more similar to naïve CD4+ T cells that are responsive to gut Ags.

T cell co-stimulation is required for TFR cell differentiation as either CD28 or ICOS deficiency leads to reduction of TFR cells (49, 113, 125). Mice with CD28 deficiency specifically in Treg cells (using Foxp3-cre) had a large reduction in TFR cells in the draining lymph node (dLN) after NP-OVA immunization (125). This is largely due to the roles of CD28 in inducing Foxp3 expression as well as TFR cell proliferation (49, 126-129). Similarly, TFR cell development is abrogated in ICOS deficient mice (113). ICOS signaling modulates the expression of Bcl6 and c-Maf in TFH cells and might play a similar role in TFR cells (130-132). Bcl6 is an essential transcription factor for TFR cells, and recent studies suggest that c-Maf is also pivotal for TFR cell differentiation (49, 50, 109, 133, 134). Bcl6 and Blimp1 reciprocally repress expression of the other factor in both TFH and TFR cells (82, 117). The regulation of TFH cell differentiation by Blimp1 is Bcl6 dependent while Blimp1 controls TFR cell differentiation independent of Bcl6 (117). One mechanism for Bcl6-independent Blimp1 activity may relate to regulation of Nfat2, which has been shown to be important for up-regulation of CXCR5 on TFR cells as well as for expression of PD-1 (105, 118). Blimp1 has been shown to repress Nfat2 expression (105), and thus Blimp1 could have a suppressive role for CXCR5 and PD-1, both of which are key genes increased in TFR cells. Increased expression of Nfat2 in Blimp1-deficient regulatory T cells could then lead to Bcl6-independent expression of CXCR5 and PD-1, and appearance of TFR–like cells (117). TFR cells were repressed by high IL-2 levels at the peak of influenza infection and this was through a Blimp1-dependent mechanism (135). IL-2 is also a negative signal for Bcl6 expression, and decreased IL-2 promotes induction of TFR cells. After the

peak anti-flu virus immune response, CD25 expression is downregulated in some Treg cells while Bcl6 is increased, leading to TFR programming (135). Thus, IL-2 is a key factor regulating TFR differentiation, promoting Blimp1 expression while repressing Bcl6 in Treg cells to preclude TFR cell development.

PD-1, which is expressed by both TFH and TFR cells, inhibits TFR differentiation and their suppressive function (49, 50, 111, 113). Sage *et al.* showed that TFR cells in *Pdcd1*-deficient mice had greater suppressive function and resulted in decreased Ab production both *in vitro* and *in vivo* (113). The exact mechanism for the increased inhibitory function of *Pdcd1*-deficient TFR cells remains unclear. At the same time, PD-1 ligand (PD-L1) is required for TFR cell generation, however it is not clear if this is a direct or indirect effect on TFR cells (54). Similarly, CTLA-4, the inhibitory receptor which binds to CD80 and CD86, limits the differentiation of TFR cells (108, 136, 137). However, restricted CTLA-4 deficiency in Treg cells contributes to not only enhanced TFR cells, but also enhanced TFH, GCB cells and Ab responses (137). One explanation is that in the absence of CTLA-4 function in Tregs, there is uncontrolled inflammation that drives higher TFH cell and GCB responses. However, since it is not clear what drives the enhanced TFH, GCB and IgE responses, a “helper” role of TFR cells cannot be completely excluded (137). Deletion of CTLA-4 results in increased IL-10 production by Tregs (138). Since IL-10 can promote GC responses (68, 139), it is possible that increased IL-10 production by TFR cells contributes to the increased GC and Ab response in CTLA-4 KO mice.

The majority of research on TFR cells has been conducted in the mouse models but a few studies have elucidated that TFR cell populations in human GCs are basically similar to TFR cells in mice (115, 140, 141). CXCR5+ TFH-like cells in blood, also known as circulating TFH (cTFH) cells, are typically used as a proxy marker for the GC TFH cell response in humans, and by assessing cTFH cell levels, a large number of genes controlling TFH cell development in humans have been categorized in patients with known specific monogenic mutations (142). Circulating TFR (cTFR) cells are also used as a correlate of the TFR cell response in humans (114, 143-146), however in contrast to TFH cells (142), only two genes that control TFR cell development and function in humans have been characterized to date: *LRBA* and *CTLA4* (146-148). Thus, more work is needed to fully understanding specific genes and pathways that regulate human TFR cells.

Suppressive functions of TFR cells

TFR cells have been described in the literature mainly as suppressors of the GC reaction and the Ab response, repressing the proliferation of TFH cells and GC B cells, and limiting the generation of Ab-secreting cells and overall Ab responses. However, the experimental approaches taken in many studies may give rise to alternative interpretations. *In vitro*, TFR cells can suppress the proliferation and cytokine production of TFH cells as well as the proliferation and Ig secretion of B cells, similar to the *in vitro* suppressive function seen with non-TFR Tregs (108, 113-115, 123, 149). *In vivo* studies have demonstrated that TFR cells, analyzed initially by depletion of total Tregs, can suppress the numbers of

GC B cells and TFH cells (49, 50, 106, 108, 113, 137). However, these studies may not represent specific effects of TFR cell depletion or physiological TFR cell function. Total Treg deletion (49, 50, 95, 108) provokes severe inflammation and causes a very broad effect on T cell responses, thus obscuring the specific functions of TFR cells. Studies using adoptive transfer of TFR cells along with other T cells into T cell deficient mice or TFH-cell deficient mice might have non-physiological effects due to the abnormal immune environment of the recipient mice (49, 50, 113, 119). Studies where TFR cell numbers are greatly enhanced due to deletion of Roquin (120), or where Tregs are forced to migrate to the B cell follicle by ectopic CXCR5 expression (118) might also lead to non-physiological suppression and/or non-specific suppression of GC responses. Mice with the *Nfat2* gene deleted in Tregs with Foxp3-cre showed a partial loss of TFR cells and augmented numbers of GC B cells, TFH cells and Ag-specific Abs after immunization (118). However, a more general loss of Treg function by loss of Nfat2 affecting TFH cell expansion cannot be discriminated from the specific effects from loss of TFR cells. *In vitro* studies of TFR cells cannot mimic the complex *in vivo* environment of the GC reaction and cannot analyze affinity selection of GC B cells. Together, a re-interpretation of the TFR cell literature helps explain why the function of TFR cells assessed using Bcl6FC mice (68, 109, 135), is strikingly different from many other studies on TFR cell function.

Nonetheless, it is clear that under some conditions, TFR cells can negatively regulate the GC reaction, and the precise mechanisms that TFR cells

use to negatively regulate the GC are unsolved questions in the TFR cell field. Treg cells can suppress immune responses by multiple known mechanisms: IL-2 consumption, secretion of inhibitory factors (IL-10, TGFβ, IL-35, granzyme B, CD39, CD73, TRAIL) and CTLA-4-mediated inhibition of TFH cell co-stimulation (150-152). Of these known suppressor factors, we can narrow down mechanisms for TFR cells based on previous data. TFH cell differentiation is inhibited by IL-2 (86, 87, 89), and IL-2 consumption by TFR cells could be predicted to help stabilize TFH cell responses. However, TFR cells have low levels (lower compared with normal Treg) of the high affinity IL-2 receptor CD25 (109), which indicates a lessened capacity to compete for available IL-2. IL-10 is unlikely to be the key suppressor factor, since IL-10 is a stimulatory or growth factor for GC B cells (68), and further, IL-10 expression by TFH cells is increased in the absence of TFR cells in Bcl6 mice (109). IL-35 is unlikely to be a TFR suppressor factor as it primarily affects T cell proliferation (153), and data with Bcl6FC mice do not indicate an effect of TFR cells on the number of TFH cells (109). Granzyme B is unlikely as a major mechanism as it is decreased in TFR cells compared to Tregs (49). Metabolic suppressor pathways such as CD39 and CD73 have not been extensively characterized and are possible effectors of suppression by TFR cells as they could potentially affect cell proliferation in the GC. In mice, TGF-β is known to stabilize TFH cell responses (154), and prevent excess TFH cell responses (155). In humans, TGF-β is required for TFH cell differentiation (93). A lack of TGF-β signaling from loss of TFR cells does not clearly explain the normal TFH cell numbers in the presence of increased TFH cytokine expression in Bcl6FC mice

(109). TRAIL is cytotoxic to follicular B cell lymphomas, which have a GC phenotype (156), but otherwise there is no data about TRAIL activity in GCs, particularly regarding TFR cells. CTLA-4 expression by TFR cells may inhibit the ability of TFH cells to receive key co-stimulation signals from GC B cells, thus limiting TFH cell and thus TFH cell-driven GC B cell expansion. Unfortunately, studies on the role of CTLA-4 function in TFR cells are difficult to interpret, as discussed above (108, 136, 137).

A recently described mechanism for TFR cells to inhibit TFH cells and the GC is secretion of a decoy IL-1 receptor that inhibits TFH cell differentiation (123). This pathway appears to be most critical during early TFH cell activation and differentiation rather than during the GC reaction itself. Furthermore, data pointing to this decoy IL-1 receptor pathway being used specifically by TFR cells to control TFH cells *in vivo* is lacking. Another potential pathway used by TFR cells to control TFH cells and GC B cells is the inhibitory receptor TIGIT (T cell immunoreceptor with Ig and ITIM domains), that is important for Treg suppressive function (109) (157). Intriguingly, the two major suppressive pathways utilized by TIGIT^{high} Tregs are IL-10 and Fgl2 (Fibrinogen-like protein 2)(157). Fgl2 is a secreted protein that binds the inhibitory IgG receptor FcγRIIB (158). As noted above, it is unlikely that loss of IL-10 from TFR cells contributes to the deregulated cytokine expression of TFH cells. Thus, Fgl2 may be a key factor used by TFR cells to regulate GC B cells. Interestingly, FcγRIIB KO mice are known to develop lupus (159), and Fgl2 KO mice develop glomerulonephritis, a pathologic manifestation of auto-Abs in

severe lupus disease also seen in IgA nephropathy (160, 161). Fgl2 KO mice have Treg cell defects, but the GC response in these mice has not been characterized (160). TIGIT+ Tregs can also affect cell activation by inducing tolerogenic DCs via CD155 (162). But currently, data on TFR cells controlling the GC via TIGIT is lacking.

In analyzing mice with deletions of Bcl6 or Stat3 specifically in the Treg lineage (Bcl6FC and Stat3FC mice, respectively), we found noteworthy differences between how TFR cells are regulated by Stat3 and Bcl6 (109, 110). While TFR cells are strongly depleted in both Bcl6FC and Stat3FC mice (109, 110), there are significant differences in the phenotype. First, in Bcl6FC but not Stat3FC mice, TFH cells produce higher levels of cytokines compared to control mice. Second, Ag-specific IgA is increased in Bcl6FC mice whereas Ag-specific IgG is increased in Stat3FC mice (109, 110). At the same time, TFH cell and GC B cell numbers are not altered in either Bcl6FC or Stat3FC mice compared to control mice (109, 110). The function of Stat3 in TFR cells is not understood. Analogous to TFH cells (163, 164), Stat3 may be important for TFR cell development by inducing Bcl6 expression in Treg cells in response to cytokines such as IL-6 and IL-21. Stat3 expression is also activated in TFR cells by the mTorc1 pathway (119). Bcl6 is required for the development of the CXCR5+PD-1+ follicular T cell phenotype, and the induction of Bcl6 by STAT factors may be essential for both TFR cell as well as TFH cell development. If this is the case though, why does deletion of Stat3 in Foxp3-expressing cells produce a different phenotype than deletion of Bcl6 in

Foxp3-expressing cells? Why are cytokines up-regulated from TFH cells in Bcl6FC mice but not in Stat3FC mice? The answers to these questions are currently unknown but are essential for fully understanding TFR cell development and function. One possible explanation for the difference is that there is a larger population of residual TFR cells in Stat3FC mice compared to Bcl6FC mice and these residual TFR cells in Stat3FC mice are enough to negatively regulate TFH cells. Thus, there is a greater deletion of TFR cells in Bcl6FC mice, leading to a more complete loss of repression by TFR cells, and thus increased TFH cell activity. The increased TFH cell cytokines in Bcl6FC mice might promote the elevated IgA response that is not seen in Stat3FC mice. In summary, in the Bcl6FC model, TFR cells repress TFH cell activity but not proliferation. Why Ag-specific IgG is increased in Stat3FC mice is unclear, but possibly the residual TFR cells in Stat3FC mice have augmented GC helper activity.

Role of TFR cells in autoimmune disease

An important area where TFR cells have a clear suppressive effect on the GC and Ab response, even in Bcl6FC mice, is in suppression of auto-Abs that drive autoimmune disease (109, 118, 125, 135, 165). This role of TFR cells in suppressing auto-Ab production was elucidated most thoroughly by Fu *et al.* who showed that Bcl6FC mice developed late-onset Sjogren's-like autoimmune disease and autoimmunity could be induced in young mice by immunizing mice with salivary gland extracts (165). The precise mechanisms for how TFR cells can suppress auto-Abs while at the same time promote the Ab response to foreign Ags

remains unexplored. One possible explanation is that since TFR cells, like Tregs, have a bias towards self-Ag recognition (53, 54, 123), they are able to inhibit self-reactive TFH cells that might develop in the GC by competing with them for recognition of self-Ags on GC B cells and binding and blocking B7 co-stimulatory receptors via CTLA-4. Little is known about the role of TFR cells in human autoimmune disease, but increased levels of circulating TFR (cTFR) cells are observed in patients with Sjogren's disease (114, 143) and systemic lupus erythematosus (144). Interestingly, an increased ratio of cTFR to cTFH cells is strongly associated with more severe disease in the case of Sjogren's syndrome (143). Whether high levels of cTFR cells simply represent the presence of active GC responses or whether cTFR cells are especially elevated in autoimmune disease is not clear. The data on Sjogren's cTFR cells is particularly hard to interpret since the cells have an immature CD25+ TFR phenotype and their relationship to GC-localized TFR cells is unclear (143).

"Helper" functions of TFR cells

Although Tregs themselves are overwhelmingly described as suppressor cells, there are several reports that Tregs can promote immune responses in certain circumstances. Under inflammatory conditions or in mice with mutations in genes that affect Foxp3 expression, a fraction of Tregs can become "ex-Tregs" and differentiate into pro-inflammatory cells (166, 167). Surprisingly, ex-Tregs can also convert into functional TFH cells in Peyer's patches (168) and in atherosclerosis (169).

The first published characterization of TFR cells in 2009 by Linterman *et al* showed that TFR cells had a key “helper” role in terms of helping TFH cells select high affinity Ag-specific B cell clones (75). In their proposed model, TFR cells restrict the outgrowth of non-Ag-specific B cell clones in the GC, presumably allowing for more efficient interaction of TFH cells with selection of specific high-affinity Abs (75). At the same time, the Linterman *et al* data can also be interpreted as showing evidence for a major helper function for TFR cells in the GC. For instance, a significant decrease in Ag-specific GC B cells is observed after total Treg depletion whereas total GC B cells increased (75). This can be interpreted as two distinct processes: 1) loss of TFR cells leads to a loss of TFR cell helper activity and thus reduced Ag-specific GC B cells, and 2) because total Tregs are depleted, there is a massive increase in GC responses to commensals and self-Ags—responses that are normally inhibited by Tregs. Even though these latter commensal-specific and self-Ag-specific GCs may be weakened by loss of TFR cell helper activity, the large number of these responses leads to a total increase in GC B cells.

A different Treg-TFH helper pathway was shown by León *et al.*, who found that Tregs are required for the normal anti-influenza TFH cell response (95). In this study, ex-Tregs were not converting into TFH cells, and León *et al.* proposed a mechanism where CD25+ Tregs take up IL-2 and limit the overall availability of IL-2, thereby promoting TFH cell differentiation (95). Importantly however, León *et al.* did not investigate loss of TFR cells (which would occur with Treg depletion) as a

mechanism for the Treg helper effect, and their data does not eliminate a helper role for TFR cells in the TFH/GC response in the virus infection system.

Because of the problems associated with deleting total Tregs and the lack of specific and robust models to deplete TFR cells *in vivo*, we developed Bcl6FC mice (109). In these mice, TFR cell development is specifically blocked without a loss in total Tregs or Treg function (109). We determined that loss of TFR cells led to a significantly decreased IgG response and that TFR cells were required to produce the highest affinity Ag-specific Abs (109). These results are consistent with a critical helper role for TFR cells in the GC. In our published results, we did not observe a loss of GC B cells or TFH cells in Bcl6FC mice despite the decreased IgG response (109). This could be due to the time-point where we analyzed the GC or the type of Ag used to induce the GC.

In 2017, Laidlaw *et al* presented very clear evidence that TFR cells can act as essential helper cells in the GC (68). In this study, mice were infected with lymphocytic choriomeninigitis virus (LCMV) and the GC and Ab response analyzed (68). Importantly, Laidlaw *et al* used Bcl6FC mice and Treg-specific IL-10 cKO mice *to* demonstrate that TFR cells are a critical source of IL-10 in the GC and that IL-10 drives the growth of GCs by promoting entry of GC B cells into the dark zone (68). In the absence of IL-10-producing TFR cells, GC B cell numbers and the LCMV-specific Ab response were decreased (68). A recent study with malaria infection in mice also showed that IL-10 was critical for the maintenance of the GC

and GC-derived Ab response (139). Overall, these recent findings strongly support the idea that IL-10-producing TFR cells have a major role in maintaining the GC reaction and thus act as “helper cells”. In our lab, we have been using Bcl6FC mice and analyzing the role of TFR cells in a food allergy model with peanut Ag. In this model, we find that TFR cells help maintain the peanut-specific GC response and IgE response. TFR cells thus appear to have a key role in allergic immune responses, represent a novel target for allergy-specific immunotherapy.

Research goals

Because of the complexity of Bcl6 regulation, the potential for regulation of IL-2 expression via glycolysis and the fact that TFH cells have an unusual state of low metabolism for effector T cells (170), we pursued the idea that Bcl6 expression and TFH cell differentiation are uniquely controlled by metabolic signals.

Additionally, due to the complexity of the interplay between transcription factors Bcl6 and Blimp1, the critical function of Bcl6 in TFH cell differentiation may be to repress the anti-TFH cell activity of Blimp1. A hybrid model is that Blimp1 and Bcl6 each control different aspects of TFH cell differentiation, so that neither factor fully controls TFH cell differentiation independent of the other. Using conditional knockout mouse models, researchers have shown that loss of Bcl6 in T cells results in complete loss of TFH cell development, while, conversely, Blimp1 cKO mice have increased TFH cell populations. Here we used conditional knockout mice doubly deficient for both Bcl6 and Blimp1 in T cells to delineate the

respective roles of these two key factors in TFH cell differentiation and PD-1 gene expression.

TFH cells have been identified as an important cell population that regulate allergic reactions and IgE through the production of IL-4. The model for TFR cell function currently is that TFR cells repress excessive TFH and GC B cell proliferation and promote the selection of high affinity B cells. Specifically, the role of TFR cells in the IgE pathway is not clear. Here, we investigated the role of TFR cells in IgE production and GC reactions using a variety of mouse models.

MATERIALS AND METHODS

Mice

C57BL/6J mice were obtained from Jackson labs and then bred in house. *Bcl6*$^{fl/fl}$ mice (88) were backcrossed to CD4-cre transgenic mice (171) and the C57BL/6 strain for at least six generations and then crossed to *Prdm1*$^{fl/fl}$ mice (172, 173). Foxp3-YFP-cre (WT), Foxp3-YFP-cre *Bcl6*$^{fl/fl}$ (Bcl6FC) mice were previously described (109). B6.129(Cg)-Foxp3tm3(DTR/GFP)Ayr/J (Foxp3-DTR), B6(SJL)-Il10ratm1.1Tlg/J (*Il10ra*$^{fl/fl}$), B6.129S7-Rag1tm1Mom/J (Rag1 KO), B6.129P2-Il10tm1Cgn/J (IL-10 KO), Tbx21-/- mice, and IL-2 knockout mice were obtained from Jackson Laboratories. Foxp3-YFP-cre *Pten*$^{fl/fl}$ (PtenFC) mice were previously described (174). Prdm1-fl/fl mice were backcrossed to Foxp3-YFP-cre transgenic mice and the C57BL/6 strain for at least six generations to generate Foxp3-YFP-cre *Prdm1*$^{fl/fl}$ (Blimp1FC) (117). MB1-cre *Bcl6*$^{fl/fl}$ (MB1-Bcl6-/-) mice were obtained from Dr. Marion Pepper (Univ. Washington). *Il10ra*$^{fl/fl}$ mice were backcrossed to MB1-cre mice to generate or MB1-cre *Il10ra*$^{fl/fl}$ (MB1-Il10ra-/-) mice. *Il10ra*$^{fl/fl}$ mice were also backcrossed to CD4-cre to generate CD4-cre *Il10ra*$^{fl/fl}$ (CD4-Il10ra-/-) mice. For Bcl6FC, Blimp1FC or PtenFC mice, Foxp3-YFP-cre only mice were used as WT controls. For MB1-Il10ra-/- or CD4-Il10ra-/- mice, *Il10ra*$^{fl/fl}$ only mice were used as controls. *Bcl6*$^{fl/fl}$ only mice were used as controls for CD4-BCL6cKO mice. Male and female mice of 6-12 weeks old were used. Mice were bred under specific pathogen-free conditions at the laboratory animal facility at Indiana University School of Medicine and were handled according to protocols approved by the

Indiana University School of Medicine Institutional Animal Use and Care Committee (IACUC). Mouse strains used in all studies are listed in Table 1.

Table 1: Experimental models: mouse strains

Strain	Original Source	Strain Code
Wild type (C57BL/6J)	The Jackson Laboratory	JAX 000664
B6.129S(FVB)-Bcl6tm1.1Dent/J (Bcl6 Flox)	The Jackson Laboratory	JAX 023727
B6.129-Prdm1tm1Clme/J (*Blimp1* Flox)	The Jackson Laboratory	JAX 008100
B6.129(Cg)-Foxp3tm4(YFP/icre)Ayr/J (Foxp3-YFP WT)	The Jackson Laboratory	JAX 016959
B6.129(Cg)-Foxp3tm3(DTR/GFP)Ayr/J (Foxp3-DTR)	The Jackson Laboratory	JAX 016958
B6(SJL)-Il10ratm1.1Tlg/J (*Il10ra* Flox)	The Jackson Laboratory	JAX 028146
B6.129S7-Rag1tm1Mom/J (Rag1 KO)	The Jackson Laboratory	JAX 002216
B6.129P2-Il10tm1Cgn/J (IL-10 KO)	The Jackson Laboratory	JAX 002251
B6.129S6-Tbx21tm1Glm/J (Tbet KO)	The Jackson Laboratory	JAX 004648

C.129P2(B6)-Il2tm1Hor/J (IL-2 KO)	The Jackson Laboratory	JAX 002229
B6.129S4-Ptentm1Hwu/J (*Pten* Flox)	The Jackson Laboratory	JAX 006440
B6.C(Cg)-Cd79atm1(cre)Reth/EhobJ (Mb1-Cre)	The Jackson Laboratory	JAX 020505
Foxp-Cre *Bcl6* $^{fl/fl}$ (Bcl6FC)	In House	N/A
Foxp-Cre *Pten* $^{fl/fl}$ (PtenFC)	In House	N/A
Foxp-Cre *Prdm1* $^{fl/fl}$ (Blimp1FC)	In House	N/A
Foxp-Cre *Prdm1* $^{fl/fl}$ *Bcl6* $^{fl/fl}$ (DKO)	In House	N/A
CD4-Cre *Bcl6* $^{fl/fl}$ (CD4-BCL6cKO)	In House	N/A
CD4-Cre *Prdm1* $^{fl/fl}$ (CD4-Blimp1cKO)	In House	N/A
CD4-Cre *Bcl6* $^{fl/fl}$ *Prdm1* $^{fl/fl}$ (CD4-dcKO)	In House	N/A
Mb1-Cre *Bcl6* $^{fl/fl}$	In House	N/A
Mb1-Cre *Il10ra* $^{fl/fl}$	In House	N/A
CD4-Cre *Il10ra* $^{fl/fl}$	In House	N/A
CD4-Cre *Bcl6* $^{fl/fl}$ IL-2KO (cIL2)	In House	N/A

Foxp-Cre *Bcl6* $^{fl/fl}$ IL-2KO (IL2BFC)	In House	N/A

Immunizations, depletion of Treg cells and Ab administration

For NP-KLH (Keyhole Limpet Hemocyanin) immunization, mice were intraperitoneally (i.p.) injected with 100 µg NP (175)-KLH (Biosearch Technologies) and were sacrificed at the indicated day. For AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide) NP-KLH immunization, 5-10 mg AICAR (Sigma-Aldrich) was mixed with 100 µg NP (175)-KLH in PBS and the mixture was injected i.p., similarly to Andris et al (176). For SRBC immunization, mice were intraperitoneally (i.p.) injected with 1 x 10^9 SRBCs (Rockland Immunochemicals) and were sacrificed at the indicated day. For food allergy challenge, mice were starved for two hours followed by feeding with 300 µl per mouse 1.5% NaHCO3 water intragastrically on indicated dates. Half hour after NaHCO3 treatment, mice were sensitized with 1 mg per mouse of peanut extract (Greer Laboratories) or ovalbumin (Sigma-Aldrich) together with 10 µg per mouse of cholera toxin (Sigma-Aldrich) (177). Mice were sacrificed on indicated days and serum, mesenteric LNs and spleens were harvested. For facial survival bleeding, about 0.3 ml blood was collected each mouse per time from the submandibular vein on indicated dates. Foxp3+ cells were deleted by administering 1 µg diphtheria toxin (DT) in PBS per mouse intraperitoneally into Foxp3-DTR mice on indicated dates. For the IL-10R blocking study, 200 µg of anti-IL-10R Ab (1B1.3A; Bioxcell) or control rat IgG1 (HRPN; Bioxcell) were injected i.p. into female C57Bl/6

mice every 3 days starting on day 1 after first intragastric (i.g.) peanut challenge. Serum was collected from the submandibular vein bleeding. Mice were subjected anaphylaxis at day 36.

Mouse T cell culture

Naïve CD4+ T cells were isolated from the spleen via isolation kit (Miltenyi Biotec). Cells were activated with plate-bound anti-CD3 (10 μg/ml; 145-2C11; Bio X Cell) and anti-CD28 (10 μg/ml; 37.51; Bio X Cell) for 72 hours in culture medium, at 1 X 10^6 cells/ml. Culture medium was glucose-free Dulbecco's Modified Eagle Medium (DMEM) media (Gibco/LifeTechnologies) supplemented with 10% dialyzed fetal bovine serum (FBS), Penicillin/Streptomycin, glutamine, non-essential amino acids, 10 mM HEPES and 55 uM β-mercaptoethanol. 10 mM glucose from a 1M stock solution (Gibco/LifeTechnologies) was added to the cultures where indicated. For IL-2 addition experiments, 100 U/ml of recombinant human IL-2 (rhIL-2) was added into culture medium as indicated. RhIL-2 was obtained from the Biological Resources Branch (BRB), Division of Cancer Treatment and Diagnosis (DCTD), National Cancer Institute-Frederick Cancer Research and Development Center (NCI-FCRDC). For primary cultures, cells were treated with indicated doses of 2-deoxyglucose (2DG; Sigma) for the duration of the culture. For secondary stimulation, after activation for 48 hours in complete medium cells were harvested and washed once with PBS. And cells were re-stimulated on plates coated with anti-CD3 and anti-CD28 with indicated medium for another 48 hours. Cells were treated with indicated doses of AICAR for 48 hours

or Compound C (Cayman Chemical) for last 24 hours during re-stimulation. EL4 cells were cultured with indicated medium for 72 hours. Cells were harvested for gene expression or flow cytometric staining. Medium supernatants were collected for indicated cytokine analysis. CD25+ Treg cells were isolated from the spleen from naïve mice via isolation kit (Miltenyi Biotec). Foxp3+YFP+ cells were further isolated by Fluorescence-activated cell sorting (FACS). Cells were stimulated for 72 hours with plate-bound anti-CD3/anti-CD28 Abs and supernatants were collected to measure IL-10 concentration.

Retrovirus production and transduction

Platinum E cells (Cell Biolabs) were grown in 10 ml of Dulbecco's modified eagle medium 1640 (DMEM 1640) with 10% FBS and 1% antibiotics in a 100 mm tissue culture dish. When confluency reached 80~90%, cells were transfected with control vector or retroviral vector containing genes encoding *Bcl6* and *Tbx21* using lipofectamine 3000 (Thermo Fisher Scientific). For transfection, 18 μg of vector, 6 μg of pCL-Eco and 25 μl of P3000 were mixed in 500 μl of Opti-MEM®I reduced-serum medium (Thermo Fisher Scientific), and 25 μl of lipofectamine 3000 was mixed in another 500 μl of Opti-MEM®I. After combining, this mixture was incubated for 10-15 mins at room temperature (RT). The mixture was gently pipetted into culture dish. After 16 hours, the media containing retrovirus was collected and changed with new fresh media. After 24 hours, the media was collected and centrifuged at 1500 rpm for 5 mins to remove cell debris. Supernatant containing retrovirus was used for retroviral transduction or stored at

-80 °C for subsequent use. For retrovirus transduction, CD4 T cells were isolated from the spleen via isolation kit (Miltenyi Biotec). Cells were activated with plate-bound anti-CD3 (10 μg/ml; 145-2C11; Bio XCell) and anti-CD28 (10 μg/ml; 37.51; Bio XCell) for 24 h at 1 X 10^6 cells/ml. Activated mouse CD4+ T cells were infected with retrovirus containing control or expressing the interested gene by centrifugation at 2300 rpm at 32^{o}C for 90 mins in the presence of 8 μg/ml polybrene (Sigma-Aldrich). For co-infection experiments, either control H2K^k or Bcl6-H2K^k-expressing (178) RVs were co-transduced with either control GFP or Tbet-GFP–expressing (179) RVs. After spin infection, supernatants were substituted with fresh medium containing 10 U/ml recombinant human IL-2. Two days later, cells were collected for flow cytometric staining, or isolated by FACS for gene expression analysis.

Human naive CD4 T cell isolation and culture

Cryopreserved human peripheral blood mononuclear cells (PBMCs) from unidentified healthy donors were thawed in a 37°C water bath, then washed twice with pre-warmed complete RPMI 1640 culture medium. Naive CD4 T cells were isolated using the “human naive CD4 T cell isolation kit” from Miltenyi Biotec (San Diego, CA) per manufacturer’s instructions. Human T cell activation beads (anti-CD3/CD28) from Invitrogen were added at 1:1 ratio. Cells were then split into different culture conditions at the density of 5x10^5 cells/mL, cultured in a 48-well tissue culture plate with either complete RPMI-1640 supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin/streptomycin, or glucose-free RPMI

supplemented with 10% dialyzed FBS and 100 U/ml of penicillin/streptomycin. Cells were treated with different concentrations of 2DG or AICAR and cultured in complete RPMI medium at 37 °C with 5% CO2 incubator for 4 days. For some cultures, cells were cultured in the presence of TFH-skewing human recombinant cytokine cocktail (1 ng/ml of IL-12, 5 ng/ml of TGF-β, and 25 ng/ml of IL-6; all from R&D Systems), Harvested cells were subjected to both flow cytometry and RT-QPCR analysis for TFH cell-specific protein markers and genes respectively.

Flow cytometry reagents

Following labelled Abs were used: anti-CXCR5 (L138D7), anti-PD-1 (29F.1A12), anti-CD4 (RM4-5), anti-FOXP3 (MF-14), anti-CXCR4 (L276F12), anti-CD86 (GL-1), anti-CD38 (90), anti-Granzyme B (GB11), anti-Klrg1 Ab (2F1/KLRG1) and anti-B220 (RA3-6B2) were obtained from BioLegend; Anti-mouse CXCR5 (2G8), GL7 (GL7), anti-Eomes (Dan11mag), anti-BCL6 (K112-91) for mouse T cells and anti-TBET (4B10) were from BD Biosciences. Anti-human CD4 (OKT4), anti-human BCL6 (BCL-UP), anti-human CD279/PD-1 (J105), anti-human CD185/CXCR5 (MU5UBEE) and fixable viability dye were purchased from eBioscience. Fixable viability dye, anti-CD38 and anti-Foxp3 (FJK-16s) Abs were from eBioscience. Fluorescent Abs for flow cytometric analysis are listed in Table 2. Annexin V Ab was obtained from BioLegend.

Table 2: Fluorescent antibodies for flow cytometric analysis

Antigen/Name	Clone	Fluorochrome	Company	Cat. No.
Mouse				
	RM4-5	PERCPCY5.5	BioLegend	100540

CD4		PECY7	BioLegend	100528
CXCR5	L138D7	PE	BioLegend	145504
CXCR5	L138D7	PECY7	BioLegend	145516
PD-1	29F.1A12	APC	BioLegend	135210
FOXP3	FJK-16s	FITC	eBioscience	53-5773-82
	MF-14	Pacific Blue	BioLegend	126410
	MF-14	FITC	BioLegend	126406
GZMB	GB11	Pacific Blue	BioLegend	515408
EOMES	Dan11mag	PERCP-	eBioscience	46-4875-82
Perforin	EBioOMAK-D	PE	eBioscience	12-9392-82
GL7	GL7	Alexa Flour	BD Pharmingen	561529
CD38	90	eFlour 450	eBioscience	48-0381-82
	90	APCCY7	BioLegend	102728
	90	Pacific Blue	BioLegend	102720
B220	RA3-6B2	PERCPCY5.5	BioLegend	103236
	RA3-6B2	APCCY7	BioLegend	103224
IgA	Ma-6E1	PE	eBioscience	12-4204-82
IgE	RME-1	PE	BioLegend	406908
IgG1	RMG-1	PECY7	BioLegend	406614
KLRG1	2F1	eFlour 450	eBioscience	48-5893-80
	2F1	PERCPCY5.5	BioLegend	138418
H2Kk	36-7-5	PE	BioLegend	114907
	36-7-5	FITC	BioLegend	114905
GATA3	16E10A23	APC	BioLegend	653806
Annexin V		PE	BioLegend	640908
BCL6	K112-91	PE-CF594	BD Biosciences	562401
CD19	6D5	Pacific Blue	BioLegend	115523
	6D5	PE	BioLegend	115508
	6D5	PECY7	BioLegend	115520
CXCR4	L276F12	PE	BioLegend	146506
CD86	GL-1	APCCY7	BioLegend	105030
Fixable Viability Dye	N/A	eFlor 780	eBioscience	65-0865-14
Fixable Viability Dye	N/A	eFluor 450	eBioscience	65-0863-14

Human				
PD-1	EH12.2H7	APC	BioLegend	329908
	eBioJ105	PE	eBioscience	12-2799-42
CXCR5	RF8B2	FITC	BD Pharmingen	558112
BCL6	BCL-UP	APC	eBioscience	17-9880-42

Cell staining for flow cytometry

Cell suspensions from mLNs and/or spleens were prepared and filtered through a 40-µm cell strainer (Fisherbrand). Cells were washed and diluted in PBS with 1% FBS and were stained with Fc block (BioXCell) for 5 min, followed by surface staining for the indicated markers. For intracellular staining, after surface markers were stained, cells were fixed and stained with Abs against transcription factors by following Foxp3 fixation kit (eBioscience) protocols. Human cells were subjected to surface staining with appropriate fluorochrome-conjugated Abs for 30 min on ice. After wash, cells were stained with viability dye for 5 min at room temperature before fixation and permeabilization 1h at room temperature followed by the intracellular staining with APC-conjugated mouse anti-BCL6 Ab 30 min in the dark. Cell events were collected on an LSR II flow cytometer (Becton Dickinson) and analyzed with FlowJo software.

Assessment of anaphylaxis

To assess anaphylaxis, 2 mg peanut extract protein without cholera toxin was administered intraperitoneally per mouse four weeks post the second immunization (day 36). Mice were monitored for 50 mins after challenge for rectal (core) body temperature change (Braintree Scientific). After 50 mins, whole blood

was collected into EDTA (Ethylenediaminetetraacetic acid) coated tubes (BD Microtainer) and hematocrit values were determined by an Element HT5 Veterinary Hematology Analyzer (HESKA). Clinical scores were evaluated at 20 to 30 mins after challenge as reported (6). Basically, 0, no clinical signs; 1, scratching around the head and nose; 2, reduced activity with increased respiration; 3, wheezing and lying prone; 4, no response after prodding and convulsion; and 5, death.

Antibody measurement and cytokine ELISAs

Ab titers of SRBC-specific or anti-NP-KLH IgG titers in serum were measured by ELISA, as previously reported (110). Briefly, 96 well Nunc-Immuno plates (Sigma-Aldrich) were coated with SRBC membrane protein or NP-KLH overnight at 4^{o}C. Wells were blocked with 1% Bovine serum albumin (BSA) and diluted serum was added and incubated at room temperature for 2 h. A peroxidase-conjugated anti-mouse IgG1 (BD), anti-IgG2b (BD), anti-mouse IgG or anti-mouse IgM Abs (Sigma-Aldrich) were used as secondary Ab. Titers of peanut-specific Ab in serum were measured by ELISA, as previously reported (180). For peanut-specific IgE, 96 well Nunc-Immuno plates (Sigma) were coated with IgE Ab (LO-ME-3, BIO-RAD) overnight at 4^{o}C. Wells were blocked with 1% BSA and diluted serum was added and incubated at room temperature for 2 hours. Peanut extract protein was labelled with biotin (Sigma-Aldrich) and added for one hour followed by adding poly-HRP streptavidin (Pierce Endogen) for half hour. For peanut-specific IgG1, 96 well Nunc-Immuno plates were coated with 5 ug/ml peanut

extract protein overnight at 4^{o}C. Wells were blocked with 1% BSA and diluted serum was added and incubated at room temperature for 2 hours. An anti-mouse IgG1 (BD Pharmingen) was used as secondary Ab with avidin-HRP. IL-2 or IL-10 concentrations of medium supernatants were measured with the mouse IL-2 or IL-10 ELISA kit from BD Biosciences, following the manufacturer's protocol. Primary and secondary Abs for ELISA are listed in Table 3.

Table 3: ELISA capture and biotinylated secondary antibodies

Capture Ab.	Clone	Final Concentration	Company	Cat. No.
IgE	R35-72	2 µg/ml	BD PH	553413
IgE	LO-ME-3	5 µg/ml	BIO-RAD	MCA419
Secondary Ab.	Clone	Dilution Factor	Company	Cat. No.
IgG1	A85-1	1: 250	BD Pharmingen	553441
IgG2b	R12-3	1: 250	BD Pharmingen	553393
IgG2a	R19-15	1: 250	BD Pharmingen	553388
IgG2c-HRP	heavy chain	1: 250	SouthernBiotech	1078-05
IgA-HRP	α-chain specific	1: 1000	Sigma-Aldrich	A4789
IgM-HRP	µ-chain specific	1: 1000	Sigma-Aldrich	A8786
IgG-HRP	whole molecule	1: 1000	Sigma-Aldrich	A9044

RNAseq

On day 36 after peanut and cholera toxin challenge, CD4+ T cells were isolated from the spleen from mice using CD4 T cell Macs isolation kit (Miltenyi Biotec). Cells were stained for TFH and TFR markers (CD4, CXCR5 and PD-1) and TFH, TFR cells were isolated by FACS. Total RNA was collected from sorted TFR cells using the RNeasy Plus Micro kit (QIAGEN) for mRNA sequencing. RNAseq and sequences analysis was performed by the Indiana University School of Medicine Center for Medical Genomics. For isolation of Klrg1+ cells, mice were immunized with SRBC and TFH cells sorted as above but anti-Klrg1 Ab (2F1/KLRG1) was also used for staining. Total RNA was collected from freshly sorted cells using the RNeasy Plus Micro kit (QIAGEN).

Gene expression analysis

Total RNA was prepared using the QIAGEN RNeasy Mini Kit following the manufacturer's protocol. Quantitative PCR (QPCR) reactions were carried using TaqMan™ Fast Advanced Master Mix (Applied Biosystems) with commercially available specific Taqman primers (Life Technologies). Samples were run in duplicates and the QPCR assays were run on the Applied Biosystems real-time QPCR machine. Mouse samples used β-tubulin (tubb5) as the reference gene, and human samples used GAPDH. Taqman probes used for qPCR are listed in Table 4.

Table 4: Taqman probes for qPCR

Mouse	
Gene	Cat. No.

Tubb5	Mm00495806_m1
Bcl6	Mm00477633_m1
Il2ra	Mm00434260_m1
Il2	Mm00434256_m1
Pdcd1	Mm01285676_m1
Cxcr5	Mm00432086_m1
Prdm1	Mm00476128_m1
Il10	Mm00439614_m1
Il4	Mm00445259_m1
Il21	Mm00517640_m1
Gzmb	Mm00442837_m1
Tbx21	Mm00450960_m1
Ifng	Mm01168134_m1
Eomes	Mm01351984_m1
Il12a	Mm00434169_m1
Human	
Gene	Cat. No.
BCL6	Hs00153368_m1
CXCR5	Hs00540548_s1
PDCD1	Hs01550088_m1
ICOS	Hs00359999_m1

RNAseq and bioinformatic analysis

RNAseq and sequence analysis was performed by the Indiana University School of Medicine Center for Medical Genomics. The RNAseq analysis was conducted using a standard workflow including sequence alignment and feature counts, then followed by differential expression analysis. First, raw reads of RNA-seq sets were mapped by using an alignment tool, STAR (181), to the UCSC Mus musculus genome (mm10) downloaded from the GENCODE (182). Then feature Counts (183) was adopted to assign uniquely mapped reads to genes according to the UCSC mm10 annotation file obtained from igenomes

(https://support.illumina.com/sequencing/sequencing_software/igenome.html). After removing low expressed genes which had less than 0.5 counts per million (CPM) in more than 12 out of 16 samples, we normalized gene expression across all samples. The dissimilarity between samples based on their gene expression profile was examined by multidimensional scaling (MDS) plot. The EdgeR software program (184) was employed to perform differential expression analysis for different comparisons. Differentially expression genes (DEGs) were determined if their p-values after multiple-test correction with FDR-adjustment were less than 0.05 and the amplitude of fold changes (FCs) were larger than 1.8.

Statistical analysis

All data analysis was done using Prism GraphPad software. Unless otherwise stated, Student t test or ANOVA with Tukey post hoc analysis were used. Only significant differences ($P < 0.05$) are indicated in Figures.

RESULTS

PART I: AMP Kinase Promotes Bcl6 Expression and Follicular Helper T Cell Differentiation in both Mouse and Human T cells

Bcl6 is regulated by glycolysis

Because of the complexity of Bcl6 regulation, the potential for regulation of IL-2 expression via glycolysis and the fact that TFH cells have an unusual state of low metabolism for effector T cells (83), we pursued the idea that Bcl6 expression and TFH cell differentiation is uniquely controlled by metabolic signals. Since blocking glycolysis during T cell activation results in an inhibition of cytokine mRNA translation, such that secretion of IFN-γ and IL-2 is markedly decreased (96), we reasoned that blocking glycolysis might play a role during TFH differentiation, by blocking inhibitory IL-2 production. Initially, as in Chang et al (96), we activated wild-type (WT) naïve CD4 T cells in glucose versus galactose medium, but we did not observe significant differences in Bcl6 expression (data not shown). However, as a control, we also activated naïve CD4 T cells in medium without added glucose or galactose, analyzed Bcl6 mRNA expression by QPCR. As shown in Fig. 5A, compared to T cells activated in 10 mM glucose, Bcl6 was increased about 10-fold when the T cells were activated in the absence of added glucose. This was accompanied by an up-regulation of Bcl6 protein, as measured by intracellular staining and flow cytometry (Fig. 5B). We tested if a transformed T cell line showed this same regulation of Bcl6 by glucose withdrawal, and so we tested the EL4 lymphoma cell line by culturing the cells with glucose or without glucose. We

observed a similar increase in Bcl6 mRNA after 48 hours in glucose-deprived conditions, and protein was increased as well as analyzed by flow cytometry (Fig. 5 C, D). We then tested the effect of the non-metabolizable glucose analogue 2DG when it was added to naïve CD4 T cells activated in DMEM medium containing glucose. As shown in Fig. 5E, Bcl6 mRNA increased over 4-fold when metabolism of the normal glucose in the culture is inhibited by 2DG. Because the effect of complete glucose deprivation (GD) on Bcl6 induction was much stronger than the effect with 2DG, we chose to focus on GD for further studies. Note, our medium contains glutamine that can be used as an alternative energy source for T cells (185, 186), though there is increased cell death and decreased proliferation in the absence of glucose. We wondered if glucose withdrawal was inhibiting T cell activation, therefore we analyzed IL-2 (*Il2*) and IL-2 receptor alpha (*Il2ra*) chain gene expression. As shown in Fig. 6A and B, in the absence of glucose, naïve CD4 T cells still become activated to transcribe levels of Il2 and Il2ra mRNAs comparable to that with glucose conditions. However, consistent with published findings that inhibition of glycolysis leads to an inhibition in cytokine translation (96), IL-2 was secreted at significantly lower levels in T cells activated under GD conditions than that under glucose conditions (Fig. 6C). Nonetheless, substantial levels of IL-2 were still secreted under GD conditions (Fig. 6C).

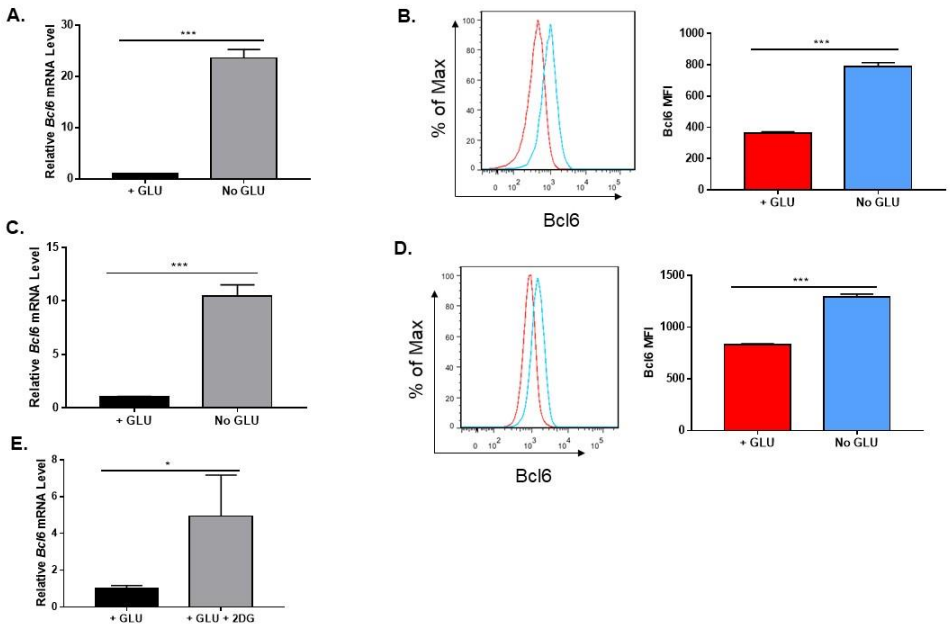

Figure 5: Induction of Bcl6 by blocking glycolysis. Naïve CD4+ T cells isolated from C57BL/6 mice or EL4 cells were cultured in complete (GLU+) or glucose (GLU-) deprivation medium. Cells were harvested for total RNA preparation or flow cytometric staining. (A) Relative mRNA expression was determined by quantitative RT-PCR. Bcl6 gene expression from isolated naïve CD4+ T cells cultured under complete or glucose deprivation medium for 72 h (n = 3, mean ± SEM). (B) Flow cytometry histogram plot for Bcl6and mean fluorescence intensity (MFI) of naïve CD4+ T cells cultured under complete or glucose deprivation medium (n = 3, mean ± SEM). (C) Bcl6 gene expression from EL4cells cultured under complete or glucose deprivation medium for 48 h (n = 3, mean ± SEM). (D) Flow cytometry histogram plot for Bcl6 and MFI of EL4 cells cultured undercomplete or glucose deprivation medium (n = 3, mean ± SEM). (E) Bcl6 gene expression from isolated naïve CD4+ T cells cultured under complete medium with or without20 mM 2DG for 72 h (n = 3, mean ± SEM). *p < 0.05, ***p < 0.001 (t test). Data are representative of 2 independent experiments of each time point with similar results.

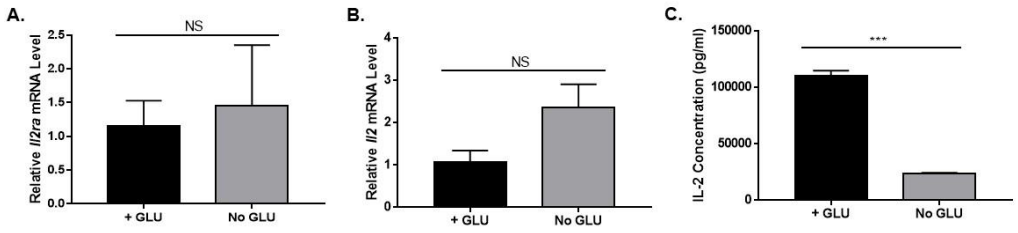

Figure 6: Effects of blocking glycolysis on IL-2 and its receptor. Naïve CD4+ T cells isolated from C57BL/6 mice were cultured in complete (GLU+) or glucose (GLU-) deprivation medium for 72 h, cells were harvested for total RNA preparation and medium supernatants were collected for ELISA analysis. (A) Relative mRNA expression was determined by QPCR. Il2ra gene expression from T cells cultured under complete or glucose deprivation medium for 72 h (n = 3, mean ± SEM). (B) Il2 gene expression from T cells cultured under complete or glucose deprivation medium for 72 h (n = 3, mean ± SEM). (C) IL-2 concentration of medium supernatants harvested from complete or glucose deprivation cell culture after 72 h (n = 3, mean ± SEM). NS (not significant, $p > 0.05$, t-test), ***$p < 0.001$ (t test). Data are representative of 2 independent experiments of each time point with similar results.

We wondered if the decreased IL-2 in the GD conditions leads to the increased Bcl6 levels. We then tested whether adding exogenous IL-2 could down-regulate the induction of Bcl6 in T cells seen with GD, by supplementing the GD cultures with high levels of recombinant human IL-2. However, the added IL-2 had no effect on Bcl6 (Fig. 7A). Since it is difficult to determine if the added human IL-2 was added to an inhibitory level equivalent to that made by T cells in glucose added conditions, we sought another approach to test this question. In order to stringently test the role of IL-2 in the GD effect on Bcl6, we used T cells from IL-2-deficient (KO) mice. When activated in vitro, IL-2 KO T cells have a higher level of Bcl6 expression than WT T cells, likely due to their inability to produce IL-2 (data not shown). We then activated naïve CD4 T cells from IL-2KO mice in plus glucose or GD conditions (Fig. 7B). When IL-2 was added to the IL-2 KO T cells activated in plus glucose conditions, Bcl6 was sharply decreased, whereas addition of a similar amount of IL-2 to IL-2 KO T cells activated in GD conditions did not significantly decrease Bcl6 expression (Fig. 7B). These data indicate that the activating effect of GD on Bcl6 expression is dominant over the inhibition by IL-2.

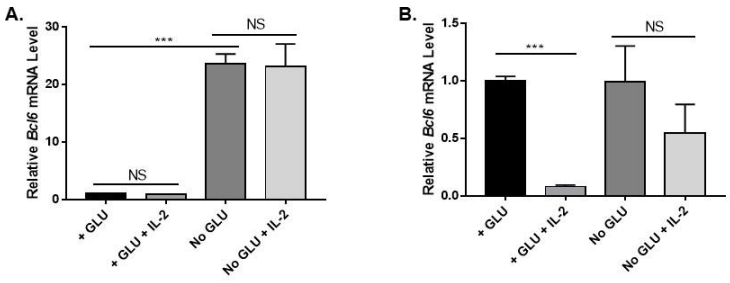

Figure 7: Up-regulation of BCL6 by glucose deprivation is insensitive to IL-2-mediated inhibition of Bcl6. Naïve CD4+ T cells isolated from C57BL/6 or IL-2 KO mice were cultured under complete (+GLU) or glucose (GLU-) deprivation media for 72 h, with or without addition of 100 U/ml recombinant human IL-2. Cells were harvested for total RNA preparation and relative mRNA expression was determined by QPCR. (A) Bcl6 gene expression from C57BL/6 naïve CD4+ T cells cultured under complete or glucose deprivation medium with or without rhIL-2 addition for 72 h (n = 3, mean ± SEM). (B) Bcl6 gene expression from IL-2 KO naïve CD4+ T cells cultured under complete or glucose deprivation medium with or without rhIL-2 addition for 72 h (n = 3, mean ± SEM). NS (not significant, $p > 0.05$, two-way ANOVA), ***$p < 0.001$ (two-way ANOVA). Data are representative of 2 independent experiments of each time point with similar results.

One pitfall of Bcl6 induction by GD is that lack of glucose during T cell activation prevents the strong T cell expansion that normally occurs during Ag stimulation *in vivo*. We therefore wondered if we could induce Bcl6 by GD in a secondary stimulation, after an initial stimulation and expansion in glucose-containing media. We thus conducted an experiment where naïve CD4 T cells were activated in plus glucose media, and then after 2 days, collected, washed, and re-activated under either plus glucose or GD conditions. As shown in Fig. 8A, Bcl6 transcript is strongly induced in secondary stimulation under GD conditions. The transcript for the activation/TFH marker PD-1 (*Pdcd1*) was also up-regulated under GD conditions along with Bcl6 (Fig. 8B), whereas the transcript for Blimp-1 (Prdm1) was not markedly increased (Fig. 8C). However, the key TFH marker CXCR5 was not increased in GD conditions compared to plus glucose. We then stained cells cultured in the secondary stimulation condition for Bcl6 (Fig. 8D, E), Tbet (Fig. 8F) and Foxp3 (Fig. 8G), and found that only Bcl6 was increased following GD. These data show that part of the TFH phenotype (Bcl6 and PD-1) can be induced during an ongoing T cell response if glucose in the immune environment is depleted while T cells are still being stimulated through the TCR, suggesting a novel pathway for TFH cell differentiation during the immune response.

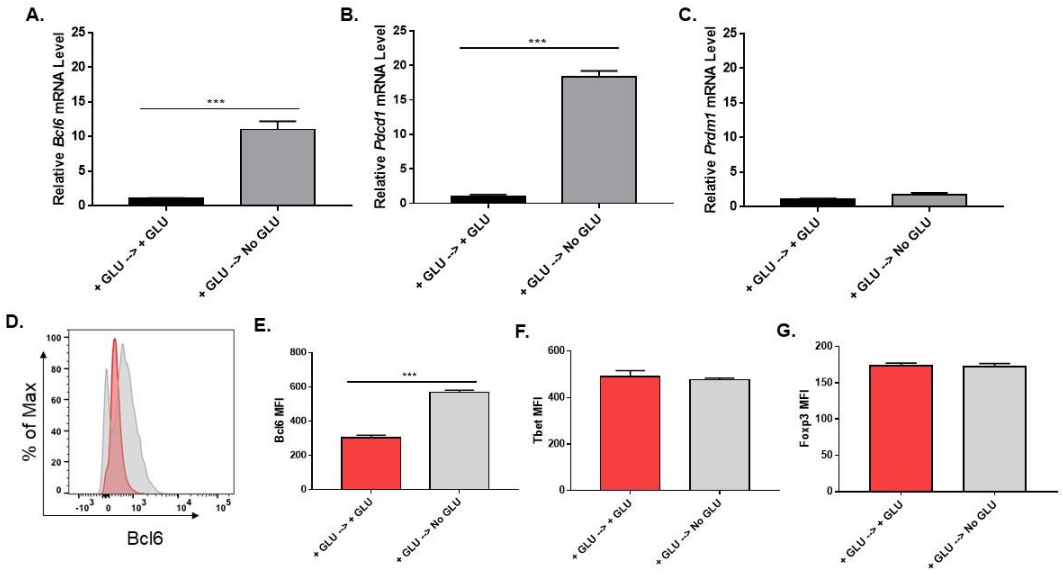

Figure 8: Up-regulation of BCL6 by glucose deprivation works with secondary re-stimulation and is specific for BCL6. Naïve CD4+ T cells isolated from C57BL/6 mice were cultured with complete media for 48 h on anti-CD3 and anti-CD28 coated plate, cells were collected and washed once with PBS and cultured under complete (GLU+) or glucose (GLU-) deprivation medium for another 48 h on anti-CD3 and anti-CD28 coated plate. Cells were harvested for total RNA preparation or flow cytometric staining. (A) Relative mRNA expression was determined by QPCR. Bcl6 gene expression from isolated naïve CD4+ T cells cultured under complete or glucose deprivation medium during re-stimulation (n = 3, mean ± SEM). (B) Pdcd1 gene expression from isolated naïve CD4+ T cells cultured under complete or glucose deprivation medium during re-stimulation (n = 3, mean ± SEM). (C) Prdm1 gene expression from isolated naïve CD4+ T cells cultured under complete or glucose deprivation medium during re-stimulation (n = 3, mean ± SEM). (D) and (E) Bcl6 MFI of naïve CD4+ T cells cultured under complete or glucose deprivation medium during re-stimulation (n = 3, mean ± SEM). (F) Tbet MFI of naïve CD4+ T cells cultured under complete or glucose deprivation medium during re-stimulation (n = 3, mean ± SEM). (G) Foxp3 MFI of naïve CD4+ T cells cultured undercomplete or glucose deprivation medium during re-stimulation (n = 3, mean ± SEM). *p < 0.05, ***p < 0.001 (t test). Data are representative of two independent experiments with similar results.

Bcl6 and the TFH response is regulated by AMP kinase

Next, we wanted to explore the signaling pathway that leads to induction of Bcl6 expression after GD. The major metabolic sensor for GD is Adenosine Monophosphate-activated Protein Kinase (AMPK), which is activated by elevated Adenosine monophosphate (AMP) or Adenosine diphosphate (ADP) levels (187, 188). Several drugs have been developed that target AMPK; compound C is a small molecule inhibitor of AMPK activity, and the AMP analog AICAR activates AMPK (189, 190). We thus tested the activity of AMPK in regulating Bcl6 expression using these compounds. As shown in Fig. 9A, naïve CD4 T cells secondarily activated under GD conditions induce strong Bcl6 expression as expected, but this up-regulation of Bcl6 can be reversed by compound C treatment. Furthermore, AICAR stimulates significant Bcl6 expression in naïve CD4 T cells secondarily activated (Fig. 9B) in the presence of glucose. Taken together, these data indicate that AMPK has key role in Bcl6 regulation in CD4 T cells. Previously, AMPK was shown to positively regulate BCL6 transcription in human endothelial cells, by a mechanism involving inactivation of PARP1 (191). To our knowledge, the data here is the first data linking BCL6 and AMPK in T cells. Previously, it was shown that AICAR could act as an adjuvant in mice, to enhance Ab responses (176), however TFH cell responses were not specifically analyzed in this study. We therefore repeated this experiment, by injecting mice with the Ag KLH either with or without AICAR. As shown in Fig. 10A, in WT mice, AICAR significantly enhanced the anti-KLH IgG titer by about 3-fold. We then tested if this Ab response was driven by TFH cells, using T cell specific Bcl6 cKO mice (80). Loss of Bcl6 in

T cells completely ablates development of TFH cells and GC B cells. When cKO mice were immunized, the overall Ab response was lower, and AICAR did not boost the Ab response to any degree (Fig 10A). These data indicate that AICAR specifically enhances the TFH cell-dependent Ab response. To more carefully analyze if AICAR affected TFH cells, we analyzed responding T cells in immunized mice at days 3, 7 and 14 after immunization with KLH or KLH plus AICAR. However, AICAR did not significantly enhance the frequency or number of TFH cells induced by immunization (Fig. 10B and data not shown), although the percentage of TFH cells was slightly higher following AICAR. However, we did observe that within the TFH cells, Bcl6 expression was significantly enhanced by AICAR (Fig. 10C). This data is consistent with our in vitro work showing that AICAR can enhance BCL6 expression. Additionally, AICAR significantly increased the number of GC B cells (Fig. 10D), which may be the result of more potent TFH cell activity due to higher Bcl6 expression, or due to a separate effect of AICAR on B cells.

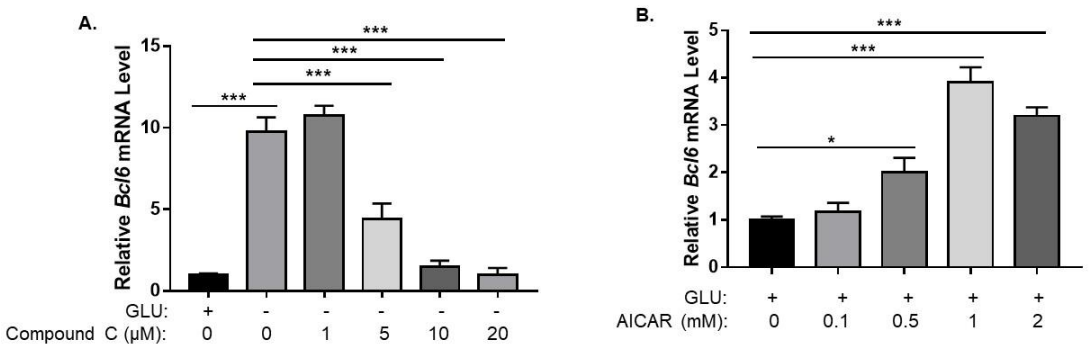

Figure 9: AMPK promotes BCL6 up-regulation, both in the presence or absence of glycolysis. Naïve CD4+ T cells isolated from C57BL/6 mice were cultured with complete media for 48 hours on anti-CD3 and anti-CD28 coated plate, cells were collected and washed once with PBS and cultured under complete or glucose deprivation medium for another 48 hours on anti-CD3 and anti-CD28 coated plate. Relative mRNA expression was determined by quantitative RT-PCR. (A) Different doses of Compound C were added to cells for the last 24 hours of re-stimulation culture. Bcl6 gene expression from isolated naïve CD4+ T cells cultured under complete or glucose deprivation medium with or without the addition of Compound C during re-stimulation (n=3, mean ± SEM). (B) Different doses of AICAR were added to cells for the 48 hours of re-stimulation culture. Bcl6 gene expression from isolated naïve CD4+ T cells cultured under complete medium with or without the addition of AICAR during re-stimulation (n=3, mean ± SEM). *$p < 0.05$, ***$p < 0.001$ (two-way ANOVA). Data are representative of two independent experiments with similar results.

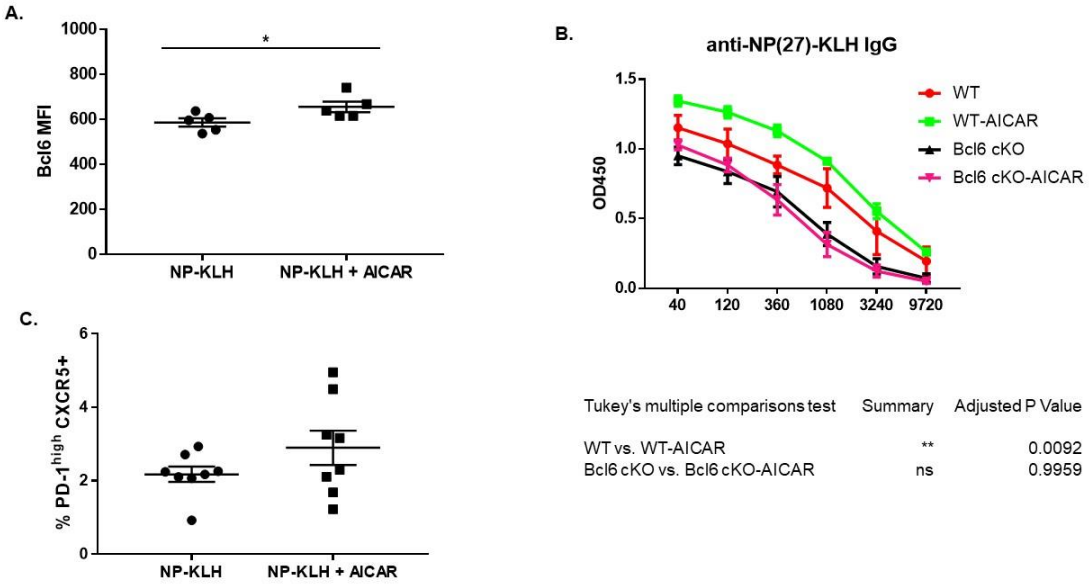

Figure 10: AICAR enhances BCL6 and TFH-dependent Ab responses in vivo. (A) C57BL/6 mice were immunized i.p. with 100 µg NP-KLH or 100 µg NP-KLH plus 10 mg AICAR and tested for in vivo Bcl6 level on day 3 post immunization. Bcl6 MFI of splenic TFH cells on day 3 post immunization (n=5, mean ± SEM). (B) WT or Bcl6 cKO mice were immunized i.p. with 100 µg NP-KLH or 100 µg NP-KLH plus 5 mg AICAR. Blood serum were harvest on day 14 post immunization for NP-KLH specific IgG ELISA analysis. The X-axis shows the dilution factors. Graphs show mean ± SEM, n = 3 (two-way ANOVA). *p < 0.05 (t test). Data are representative of two independent experiments with similar results.

AMPK regulates BCL6 and TFH markers in human T cells

Since conditions for TFH cell differentiation have been more clearly characterized in human T cells compared to mice (93), we were curious if our findings with regulation of mouse Bcl6 by AMPK translated to human T cells. We therefore isolated naïve CD4 T cells from human peripheral blood mononuclear cells (PBMC), stimulated them in vitro with anti-CD3/anti-CD28 beads, and added reagents that modulate AMPK activity. We found that 2DG potently up-regulated both BCL6 and CXCR5 in human CD4 T cells (Fig. 11A, B). In fact, human T cells were extremely sensitive to 2DG compared to mouse cells, such that only 1.25 mM 2DG was effective at increasing BCL6, whereas much higher concentrations of 2DG were required to induce Bcl6 in mouse T cells (Fig. 5E). Nonetheless, this result indicated a similar regulatory pathway might exist for both mouse and human T cells. We then tested AICAR in the human CD4 T cell culture system. Similar to mouse T cells, AICAR was effective at significantly inducing BCL6 expression, both at the mRNA (Fig. 11C) and protein (Fig. 11E) level. Unlike with mouse T cells, AICAR induced significant up-regulation of the TFH markers CXCR5 (Fig. 11D) and PD-1 (Fig. 11F). Strikingly, AICAR was more effective at inducing transcription of BCL6 (Fig. 11C) and CXCR5 (Fig. 11D) than when the CD4 T cells were cultured in characterized TFH cell conditions (93). These data indicate that mouse and human T cells share a common AMPK pathway for inducing BCL6, however it also appears that human CD4 T cells are also more prone to develop into TFH–like cells than mouse CD4 T cells when this pathway is activated.

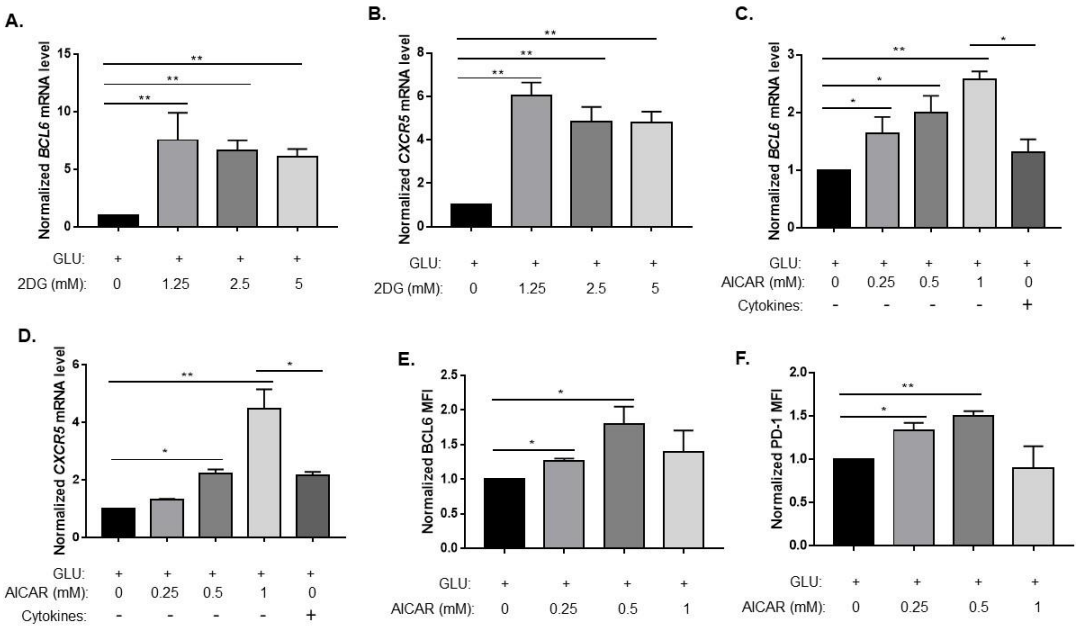

Figure 11: The AMPK pathway promotes TFH-like differentiation in human CD4 T cells. Human naïve CD4 T cells were treated with 2DG (A, B) or AICAR (C-F) at the concentrations indicated, in complete (GLU+) RPMI medium containing anti-CD3/CD28 activation beads, cultured for 4 days. In (C, E), cells marked “Cytokines” were cultured with TFH skewing cytokines (IL-6, IL-12, TGFβ). (A, B, C, E) The expression levels of TFH genes (*BCL6*, *CXCR5*) were analyzed by QPCR, and normalized to the levels of GAPDH. (D, F) AICAR-treated cells were subjected to surface and intracellular staining with Abs to BCL6 and PD-1. Data are the normalized results from 3 individual T cell samples and preparations. Graphs show mean ± SEM, n = 3 (two-way ANOVA). *p < 0.05 (t test).

PART II: Bcl6 Promotes Follicular Helper T-cell Differentiation and PD-1 Expression in a Blimp1-independent Manner in Mice.

TFH cells but not TFR cells are dependent on Bcl6 for their development.

The transcription factors Bcl6 and Blimp1 have opposing roles in the development of the follicular helper T (TFH) cells: Bcl6 promotes and Blimp1 inhibits TFH-cell differentiation. Similarly, Bcl6 activates, while Blimp1 represses, expression of the TFH-cell marker PD-1. However, Bcl6 and Blimp1 repress each other's expression, complicating the interpretation of the regulatory network. Here we sought to clarify the extent to which Bcl6 and Blimp1 independently control TFH-cell differentiation by generating mice with T-cell specific deletion of both Bcl6 and Blimp1 (double conditional KO [dcKO] mice). Our results indicate that Bcl6 promotes both TFH cell differentiation and PD-1 expression by pathways essentially independent of Blimp1.

To analyze TFH cell differentiation when both Bcl6 and Blimp1 were deleted specifically in T cells, we generated CD4-cre *Bcl6*$^{fl/fl}$ *Prdm1*$^{fl/fl}$ (dcKO) mice. These mice were healthy and T cell development was normal (data not shown). We immunized the mice with SRBC, and after 14 days, analyzed TFH cells in spleen (Fig. 12A and Fig. 13A). Control (Con) mice were *Bcl6*$^{fl/fl}$ *Prdm1*$^{fl/fl}$ without CD4-cre. Bcl6 cKO and Blimp1 cKO mice were also analyzed. Compared with Con, TFH cell populations were increased two-fold in Blimp1 cKO mice, while TFH cells were completely absent in Bcl6 cKO and dcKO mice. These data indicate that Bcl6

is required for TFH cell development even in the absence of Blimp1-mediated repression. Next, we analyzed the development of T follicular regulatory cells (TFR cells) (49, 50, 106, 109) after SRBC immunization (Fig. 12A and Fig. 13C, D). Blimp1 cKO mice have a sharply enhanced TFR cell population compared with Con mice, while TFR cells are nearly absent in Bcl6 cKO mice. However, TFR cells are significantly higher in dcKO mice than Bcl6 cKO mice, indicating that Blimp1 represses TFR cells independent of Bcl6, and that TFR cells and TFH cells have somewhat different modes of development. We also analyzed GC B-cells by flow cytometry in the same sets of mice (Fig. 12B and Fig. 13E, F), and GC B-cell development paralleled TFH cell development in all four mouse lines. SRBC-specific IgG titers followed the levels of germinal center B (GCB) cells (Fig. 14). We analyzed TFH cells, TFR cells and GCB-cells 7 days after SRBC immunization, and observed the same pattern as after 14 days (data not shown).

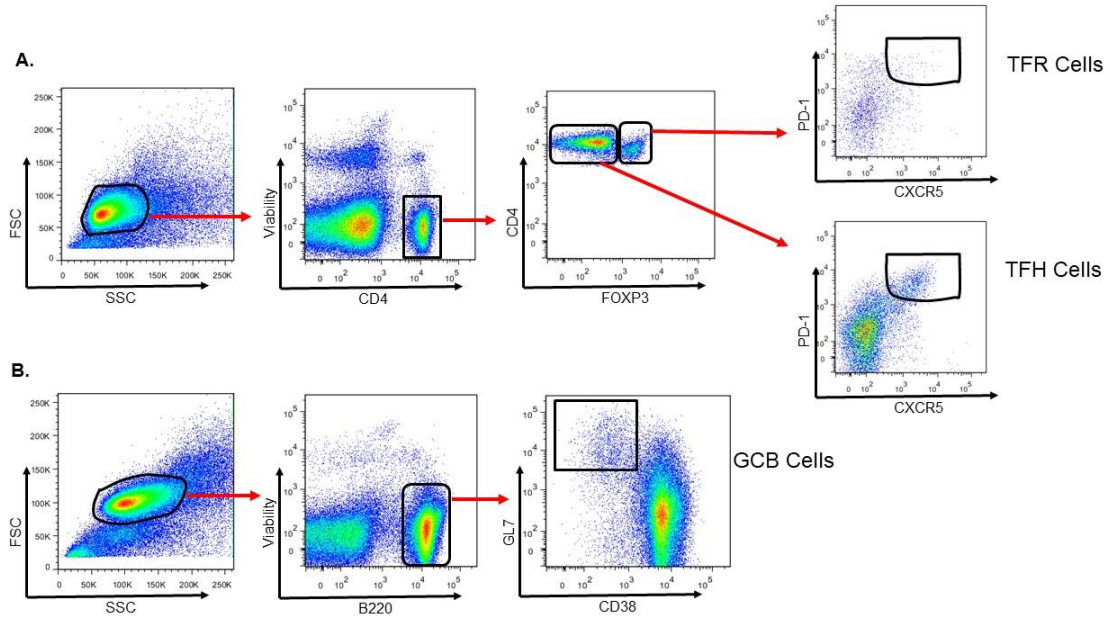

Figure 12: Gating strategies for TFH, TFR and germinal center B cells. Splenic single cell suspensions were admitted to flow cytometry machine, lymphocytes were first gated out using SSC and FSC. Then viable CD4+ T cells were gated and further gated as Foxp3+ or Foxp3- populations. TFH or TFR cells were gated from Foxp3- or Foxp3+ CD4+ T cells with CXCR5+PD-1+. GCB cells were gated using B220+ and GL7+CD38- markers.

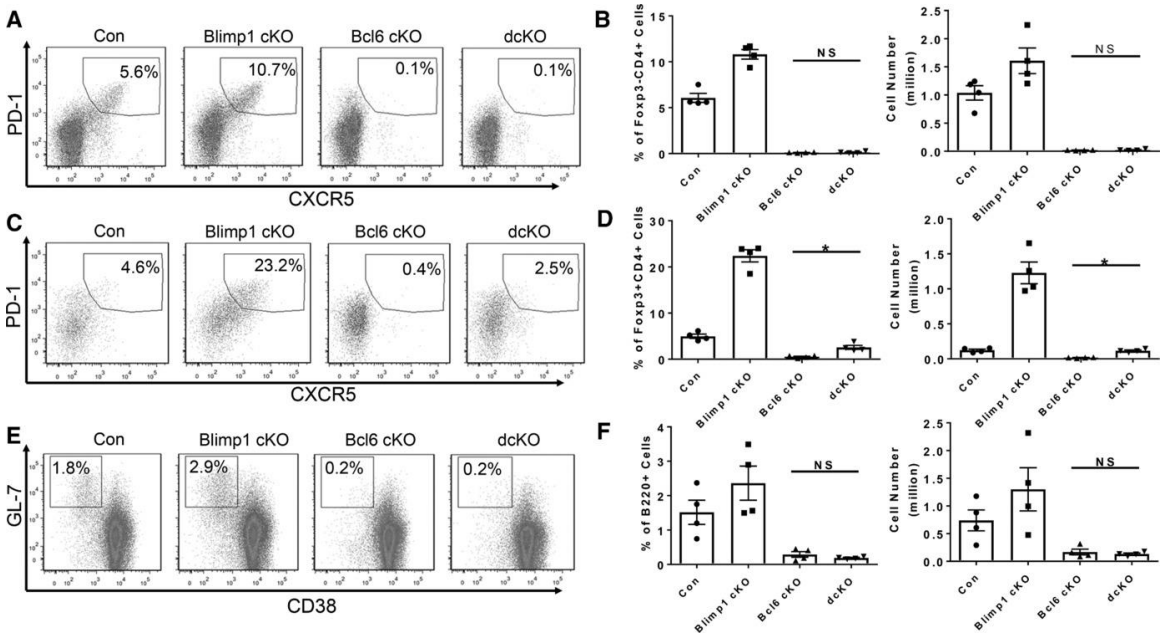

Figure 13: Regulation of TFH cells, TFR cells and germinal center B cell by Bcl6 and Blimp1. Control, Blimp1 cKO, Bcl6 cKO and dcKO mice were immunized with SRBC by i.p. injection. Fourteen days post-immunization (dpi), spleens were isolated for flow cytometric analysis. TFH cells are defined as Foxp3−CXCR5^{hi}PD-1^{hi}. TFR cells are defined as Foxp3+CXCR5^{hi}PD-1^{hi}. GC B cells are defined as B220+CD38^{lo}GL-7^{hi}. (A) Representative flow cytometry plots, gated on FoxP3– CD4+cells. (B) TFH-cell percentage within Foxp3− CD4+ T cells and absolute TFH-cell number per spleen. (C) Representative flow cytometry plots, gated on Foxp3+ cells. (D) TFR-cell percentage in Foxp3+CD4+ T cells and absolute TFR-cell number per spleen. (E) Representative flow cytometry plots. (F) GCB-cell percentage in B220+ B cells and absolute GCB-cell number per spleen. Flow cytometry plots are from a single experiment representative of 2 experiments with 16 total mice per experiment. Data in graphs are shown as mean ± SEM, $n = 4$ with each symbol representing a single mouse. NS = not significant, $p > 0.05$, $*p < 0.05$ (two-way ANOVA). Data are representative of 2 independent experiments with similar results with 16 total mice per experiment.

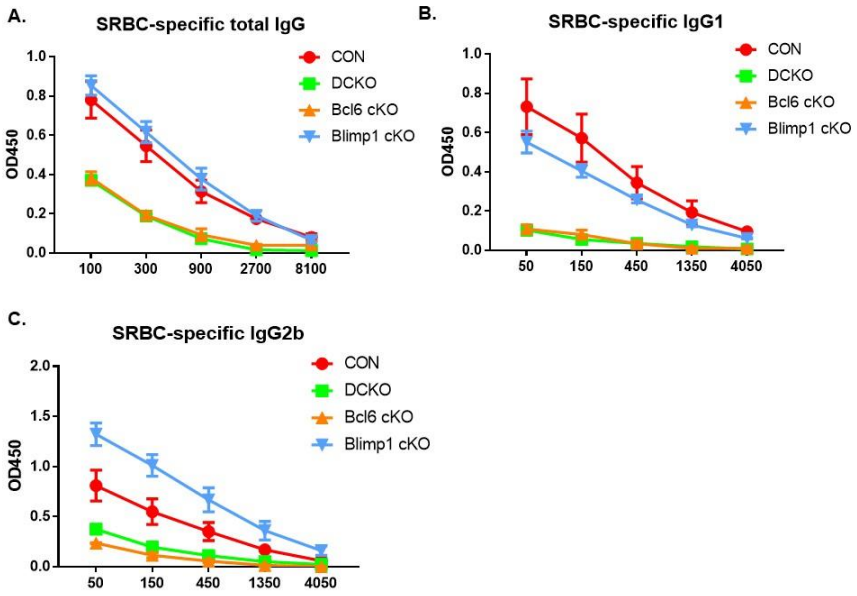

Figure 14: Antigen-specific IgG titers correlate with GC B cell responses. Control (Con), Blimp1 cKO, Bcl6 cKO and dcKO mice were immunized with SRBC by i.p. injection. At 14 dpi, blood serum samples were harvested for Ab analysis. (A) SRBC-specific total IgG, (B) SRBC-specific IgG1, (C) SRBC-specific IgG2b titers. X-axis shows the dilution factors. Graphs show mean ± SEM, n = 4 (two-way ANOVA). Data are representative of two independent experiments with similar results.

Bcl6 controls PD-1 expression independent of Blimp1

Bcl6 and Blimp1 have opposing roles in PD-1 expression (104, 105), and we therefore analyzed PD-1 expression in immunized Con, Bcl6 cKO, Blimp1 cKO and dcKO mice (Fig. 15). The average level of PD-1 expression measured by MFI in total CD4 T cells showed very high PD-1 in CD4 T cells from Blimp1 cKO mice (Fig. 15A and Fig. 16A), consistent with Blimp1 repressing PD-1 expression. Since PD-1 expression in dcKO CD4 T cells is much lower than in Blimp1 cKO CD4 T cells, this indicates a positive role for Bcl6 in PD-1 regulation. Since the PD-1 levels on total CD4 T cells are skewed by the presence of PD-1^{high} expressing TFH cells, we analyzed PD-1 expression on PD-1^{+} non-TFH cells (Fig. 15B and Fig. 16B). Loss of Blimp1 again increased PD-1 in non-TFH cells, whereas PD-1 in Bcl6 cKO and dcKO non-TFH cells was similar as control (Con) levels. These data indicate that the higher PD-1 in Blimp1 cKO non-TFH cells requires Bcl6 expression, but did not answer whether Bcl6 could regulate PD-1 independently of Blimp1. To address this question, we used retroviruses (RVs) to express Bcl6 in activated CD4 control and dcKO T cells, then assessed PD-1 expression. As shown in Fig. 15C and Fig. 16C, Bcl6 significantly augmented PD-1 expression as measured by MFI, in both control and dcKO T cells, showing clearly that Bcl6 could activate PD-1 expression independent of Blimp1. Indeed, Bcl6 induced higher expression of the *Pdcd1* gene (Fig. 17A), whereas a known Bcl6 target, *Il10* (80, 101) was strongly repressed by Bcl6 RV (Fig. 17B). Thus, Bcl6 can both activate and repress gene expression, independently of Blimp1. Since Blimp1 has been shown to positively regulate IL-10 (192), our data also rules out a mechanism of IL-10 control

where Bcl6 acts on IL-10 by repressing Blimp1 and causing decreased *Il10* transcription, and shows that Bcl6 is a direct repressor of *Il10* expression.

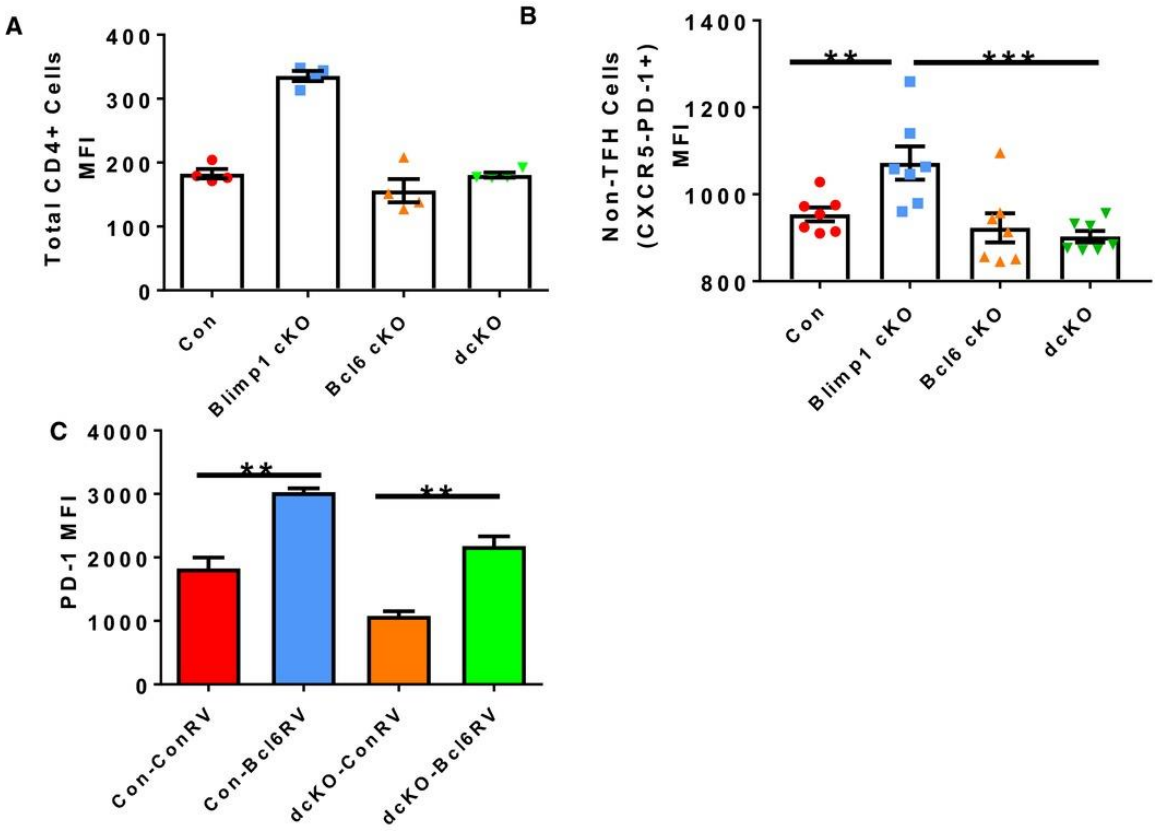

Figure 15: Control of PD-1 expression by Bcl6 and Blimp1. Control, Blimp1 cKO, Bcl6 cKO and dcKO mice were immunized with SRBC by i.p. injection. At 14 days dpi, spleens were isolated for flow cytometric analysis. (A) PD-1 MFI of total CD4+ T cells at 14 dpi ($n = 4$, mean ± SEM). (B) PD-1 MFIs of CD44+ CXCR5− PD-1+ non-TFH cells. (C) Control and dcKO CD4+ T cells were infected with Bcl6-expressing and control retroviruses (RVs). $^{**}p < 0.01$ (two-way ANOVA). Each symbol in graphs represents one mouse. Data are representative of 2 independent experiments with similar results with 16 total mice per experiment.

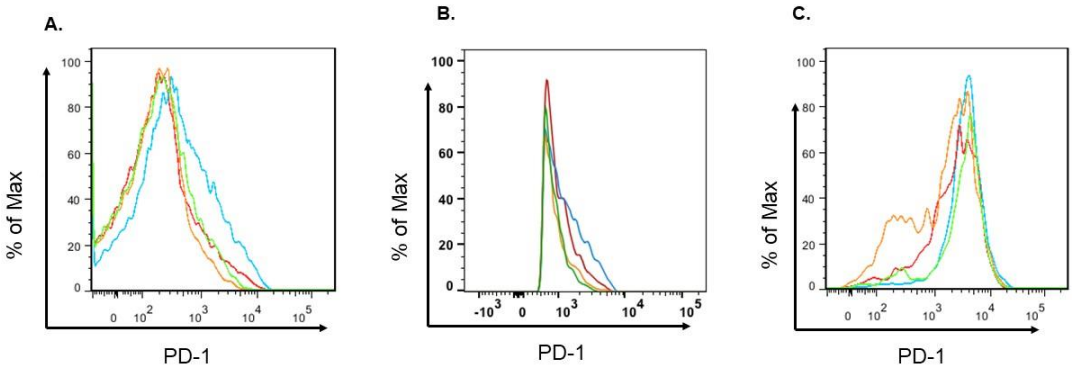

Figure 16: Control of PD-1 expression by Bcl6 and Blimp1 (2). Control, Blimp1 cKO, Bcl6 cKO and dcKO mice were immunized with SRBC by i.p. injection. At 14 days dpi, spleens were isolated for flow cytometric analysis. (A) histogram plot showing representative PD-1 expression and (B) PD-1 MFIs of CD44+ CXCR5- PD-1+ non-TFH cells at 14 dpi (n=7, mean ± SEM). (C) PD-1 MFIs of control and dcKO CD4+ T cells infected with Bcl6-expressing and control RVs. All color codes correspond to Figure 15. **p < 0.01, ***p < 0.001 (two-way ANOVA). Each symbol in graphs represents one mouse. Data are representative of two independent experiments with similar results.

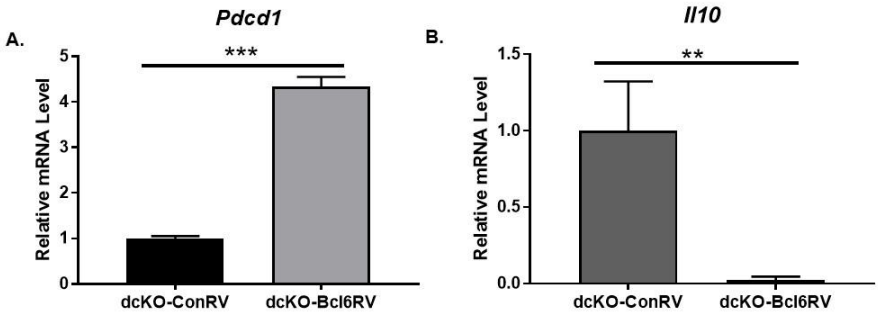

Figure 17: Bcl6 regulates IL-10 and PD-1 independent of Blimp1. Control and dcKO CD4+ T cells were infected with Bcl6-expressing and control retroviruses (RVs). (A) Relative mRNA expression was determined by quantitative RT-PCR. *Pdcd1* gene expression from isolated dcKO-ConRV and dcKO-Bcl6RV infected cells (n=3, mean ± SEM). (B) IL-10 (*Il10*) gene expression from isolated dcKO-ConRV and dcKO-Bcl6RV infected cells after 5 hours of re-stimulation in anti-CD3 and anti-CD28 coated wells (n=3, mean ± SEM). **p < 0.01 ***p < 0.001 (t test).

A T-bet-dependent mechanism for the activation of PD-1 expression by Bcl6

We next sought to find the mechanism for how Bcl6, a transcriptional repressor, could promote *Pdcd1* mRNA expression independent of Blimp1. One explanation is that Bcl6 represses the transcription of microRNAs that silence PD-1 expression (193). We therefore tested the role of microRNAs in the induction of PD-1 by Bcl6, and found that Bcl6 RV could still up-regulates PD-1 in *Dicer* cKO T cells, which are unable to generate microRNAs (Fig. 18). We then sought other pathways. T-bet is a transcriptional repressor of PD-1 gene expression (194), and Bcl6 is a negative regulator of T-bet (195). Therefore, we wondered if a T-bet-Bcl6 pathway could play a role in PD-1 expression in CD4 T cells. We tested this idea by transducing primary mouse CD4 T cells with T-bet-expressing RV, with and without Bcl6 RV. As shown in Fig. 19A, T-bet RV can significantly repress PD-1 expression compared with control RV, whereas addition of Bcl6 RV allows for PD-1 activation even in the presence of T-bet RV. To further investigate this pathway, we analyzed PD-1 expression in *Tbx21*$^{-/-}$ (Tbx21 KO or T-bet-deficient) CD4 T cells and as expected, we saw higher PD-1 in T-bet KO T cells compared with wild-type T cells (Fig. 19B). Notably, Bcl6 RV was able to strongly activate PD-1 expression in wild-type T cells (~50% increase in MFI) but Bcl6 RV was only able to activate PD-1 relatively weakly in T-bet KO T cells (~20% increase in MFI) (Fig. 19B and C). These data indicate that a major pathway for the up-regulation of PD-1 expression by Bcl6 is by counteracting the repressive action of T-bet on PD-1 expression.

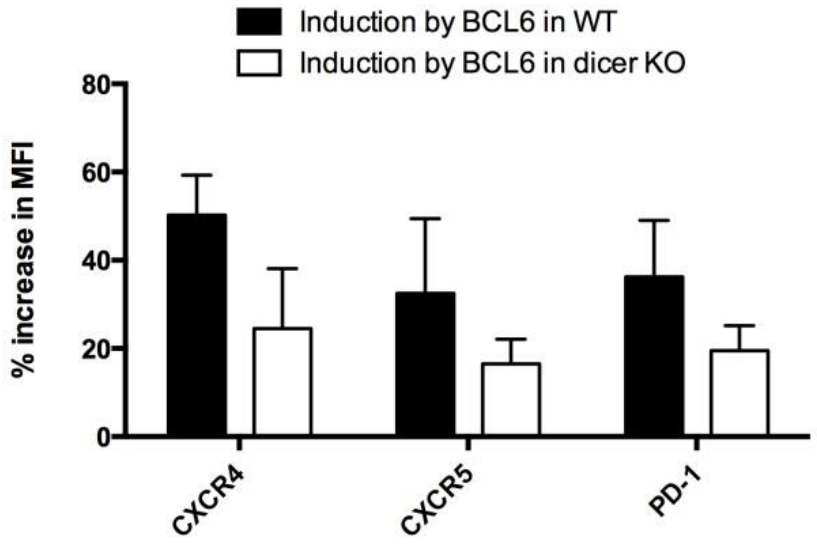

Figure 18: BCL6 can augment CXCR4, CXCR5 and PD-1 expression in the absence of microRNA. Naïve CD4 T cells were taken from wild-type and CD4-cre dicer-fl/fl mice, activated with CD3/CD28 Abs then infected with GFP control or GFP/BCL6-expressing retrovirus. After 2 days in culture, cells were stained for CXCR4, CXCR5 and PD-1 and expression in GFP+ cells analyzed by flow cytometry. Graph shows the % increase in expression induced by BCL6 in either wild-type or dicer KO T cells, as well as the general increase in expression in wild-type T cells versus dicer KO T cells. Data from 4 separate experiments was averaged for the graph. (Data generated by Hao Wu).

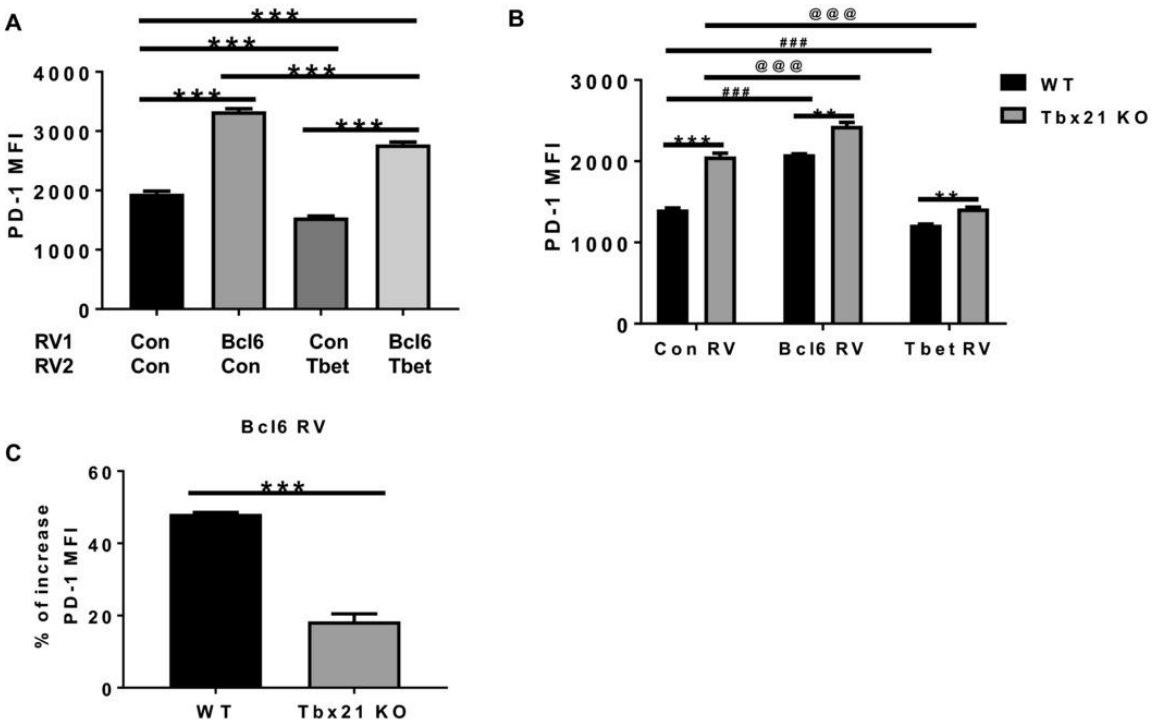

Figure 19: Control of PD-1 expression by Bcl6 and Tbet. Total CD4+ T cells from wild-type (WT) or Tbx21 cKO mice were co-infected with Bcl6-H2K^k-expressing and T-bet-GFP-expressing and control RVs. PE-labeled anti-H2K^k Ab was used to detect H2K^k expressing cells. Double GFP+PE+ cells were gated on for analysis. (A) PD-1 MFI of total CD4+ T cells from WT mice after control, Bcl6, T-bet or Bcl6 plus T-bet RV co-infection (n = 4, mean ± SEM). (B) PD-1 MFI of total CD4+ T cells after control, Bcl6, T-bet or Bcl6 plus T-bet RV co-infection. Statistical designations: * compares WT to Tbx21 KO, $^{\#}$ compares WT with the different RV infections, $^{@}$compares Tbx21 KO with the different RV infections. (C) Percentage of increase of PD-1 MFI of total CD4$^+$ T cells after control or Bcl6 RV infection in same experiment as (B). ***, $^{@@@, \#\#\#}$ = $p < 0.001$, ** = $p < 0.01$ (t-test). Data are representative of two independent experiments with similar results, and with four mice per condition per experiment.

In summary, we have clarified several aspects of Bcl6 control over TFH cell and TFR cell differentiation (see model in Fig. 20). Specifically, we have found that: (i) Blimp1 primarily represses TFH cell differentiation by acting on Bcl6, (ii) Blimp1 represses TFR cell differentiation through both Bcl6-dependent and Bcl6-independent pathways and (iii) Bcl6 promotes PD-1 expression by a novel Blimp1-independent mechanism involving T-bet inhibition.

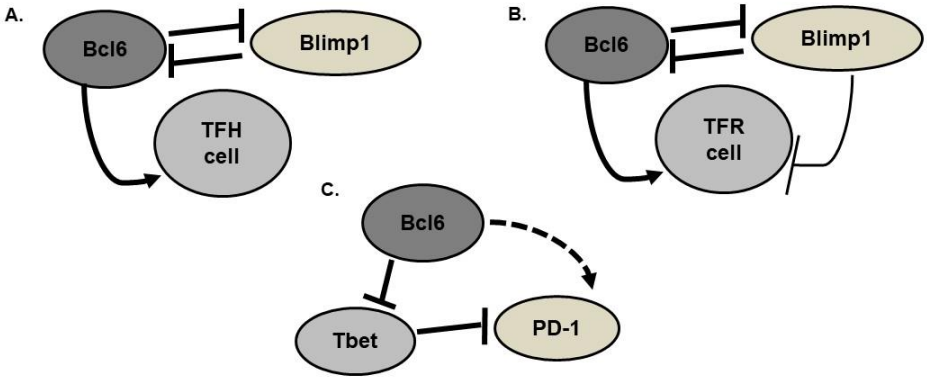

Figure 20: Models for regulation of TFH cells, TFR cells and PD-1 by Bcl6, Blimp1 and Tbet. (A) Regulation of TFH cells by Bcl6 and Blimp1, (B) Regulation of TFR cells by Bcl6 and Blimp1, (C) Regulation of PD-1 by Bcl6 and Tbet. Arrow = positive regulation, T-end line = negative regulation, dotted line = indirect regulation.

PART III: Follicular Regulatory T Cells are Necessary for and Promote Antigen-Specific IgE Production in a Food Allergy Model.

TFR cells have been shown to suppress the proliferation of TFH cells and GC B cells, and can also facilitate the selection of high affinity B cell clones in the GC. Here, we used a peanut Ag food allergy model in mice to examine the role of TFR cells in controlling the production of Ag-specific IgE and IgE-mediated anaphylaxis.

Production of antigen-specific IgE in a food allergy model is dependent upon both TFR cells and the GC reaction.

To study the role of TFR cells in regulating IgE production and IgE responses, we used our Bcl6FC mouse model, in which TFR cells do not develop (109), and a widely-used model of food allergy induced by i.g. challenge of peanut protein plus cholera toxin (PCT)(Fig. 21A)(177). In this food allergy model, high levels of peanut-specific IgE are induced and maintained in circulation for weeks. As shown in Fig. 21B, 4 weeks after the challenge period, WT mice produced substantial levels of peanut-specific IgE. Our initial expectation was that IgE would be produced at higher levels in the absence of TFR cells, however we found that the IgE response was essentially lost in Bcl6FC mice after 4 weeks (Fig. 21B). Peanut-specific IgG1 was significantly decreased in the Bcl6FC mice but was easily detectable (Fig. 21B). We then analyzed the time course of serum peanut-specific IgE and IgG1 induction and maintenance in the PCT model (Fig. 21C, D).

We observed that peanut-specific IgE was induced to high levels one week after the second PCT challenge and then slowly decreases over several weeks, whereas in Bcl6FC mice, the initial induction of IgE is severely blunted and then fades to undetectable levels at two weeks after PCT challenge. In contrast, peanut-specific IgG1 was strongly induced and maintained at high levels for weeks after the PCT challenge in both WT and Bcl6FC mice, even though the levels were significantly lower in the TFR-deficient Bcl6FC mice. To assess the physiological relevance of peanut-specific IgE levels at day 36, we induced anaphylaxis by i.p. injection of peanut protein alone (Fig. 21E). A strong anaphylaxis response was induced in PCT challenged WT mice, while anaphylaxis was dramatically weaker in Bcl6FC mice. We then tested if the induction of peanut-specific IgE was dependent on the GC reaction by performing PCT challenge of CD4-cre Bcl6-flox mice which lack TFH and GCB cells (103). We observed that the anti-peanut IgE response was completely ablated in these mice although some anti-peanut IgG1 was still produced (Fig. 21F). Similar results on GC dependence were observed when we tested Mb1-cre Bcl6-flox mice by PCT challenge (Fig. 22 A, B). CD4-cre Bcl6-flox mice showed no signs of anaphylaxis when challenged i.p. with peanut protein (Fig. 21G), confirming the IgE dependence of the anaphylaxis and showing the strict dependence of Ag-specific IgE responses on the GC reaction. Similar results showing Ag-specific IgE dependence on GC responses and TFR cells were observed when Ovalbumin was substituted for peanut protein in the food allergy model (Fig. 22 C, D), showing that these results were not unique to peanut as an Ag. Overall these data indicate that in this food allergy model, the development of

peanut-specific IgE requires the GC reaction and that TFR cells play a helper rather than a suppressor role in IgE responses. Our data also imply that TFR cells act within the GC to regulate IgE.

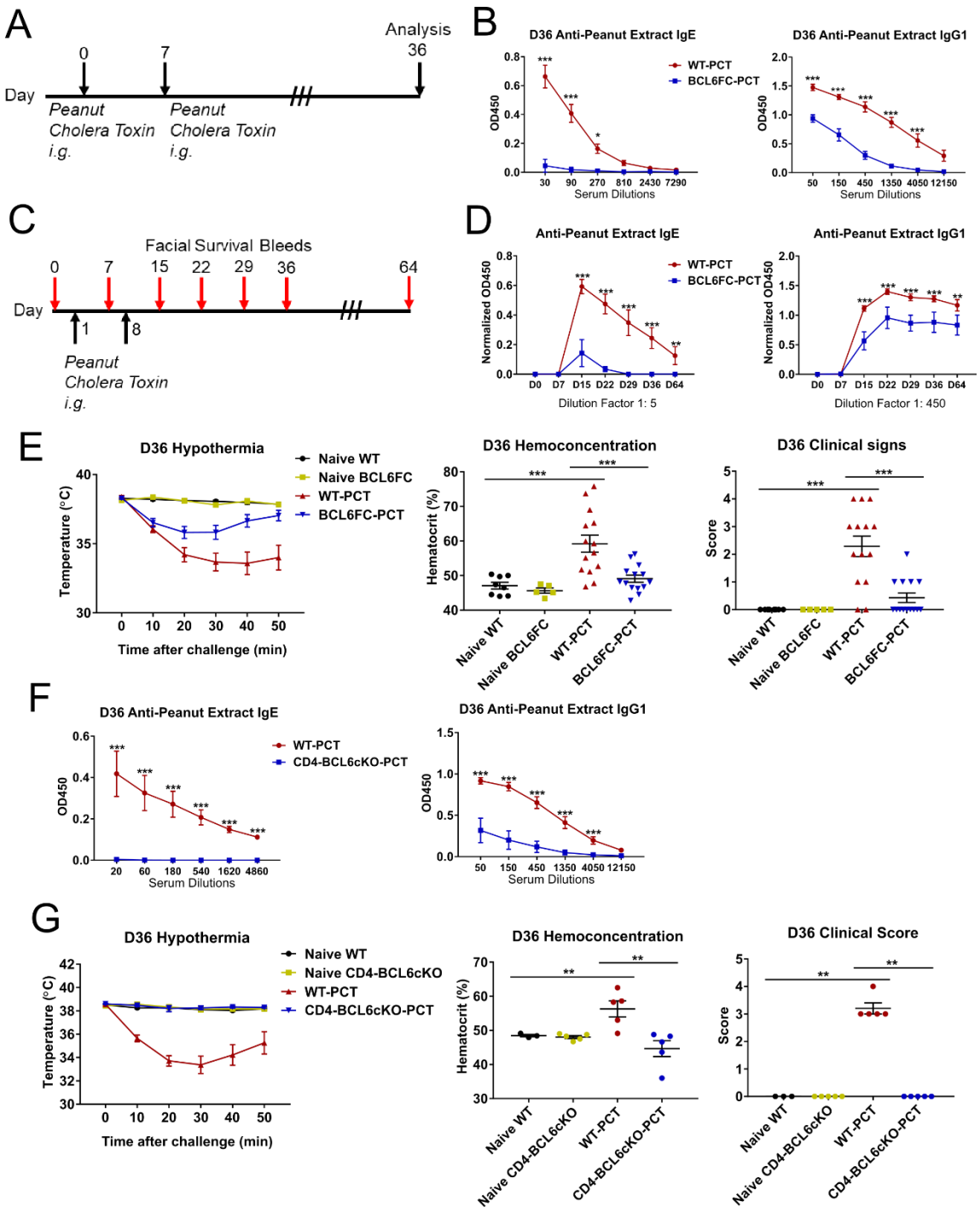

Figure 21: Lack of TFR cells in a food allergy model leads to loss of antigen-specific IgE and decreased anaphylaxis responses.

Figure 21: Lack of TFR cells in a food allergy model leads to loss of antigen-specific IgE and decreased anaphylaxis responses. The food allergy model uses two i.g. challenges in mice of peanut protein plus cholera toxin (PCT) 7 days apart, then bleeds at different time-points after challenge. (A) shows the day 36 (D36) time-course, where serum is tested 28 days after the last challenge for peanut-specific Abs. (B-D) Control Foxp3-cre alone (WT) mice and Foxp3-cre Bcl6-flox (Bcl6FC) mice were challenged as shown in (A) and D36 serum was tested for peanut-specific IgE and IgG1 (B) or at various time-points during and after challenge (C-D). (E) WT and Bcl6FC mice challenged as in (A) were analyzed for anaphylactic responses at D36. Naïve unchallenged WT and Bcl6FC mice were used as negative controls. For induction of anaphylaxis, female mice were injected i.p. with 2 mg of peanut protein at D36, then monitored for drop in body temperature (hypothermia) over the course of 50 minutes (min). Clinical signs were assessed 20 to 30 min after injection. After 50 min, blood was taken from the mice and percent hematocrit (hemoconcentration) tested. (F-G) Control *Bcl6*$^{fl/fl}$ alone (WT) mice and CD4-cre Bcl6-flox (CD4-Bcl6 cKO) mice were challenged as shown in (A). (F) D36 serum was tested for peanut-specific IgE and IgG1. (G) Female mice were tested for anaphylaxis as described in (E). Naïve unchallenged WT and CD4-Bcl6 cKO mice were used as negative controls. P values were calculated by t test or ANOVA where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. N = 4 - 6 mice, and each experiment was repeated at least 2 or 3 times.

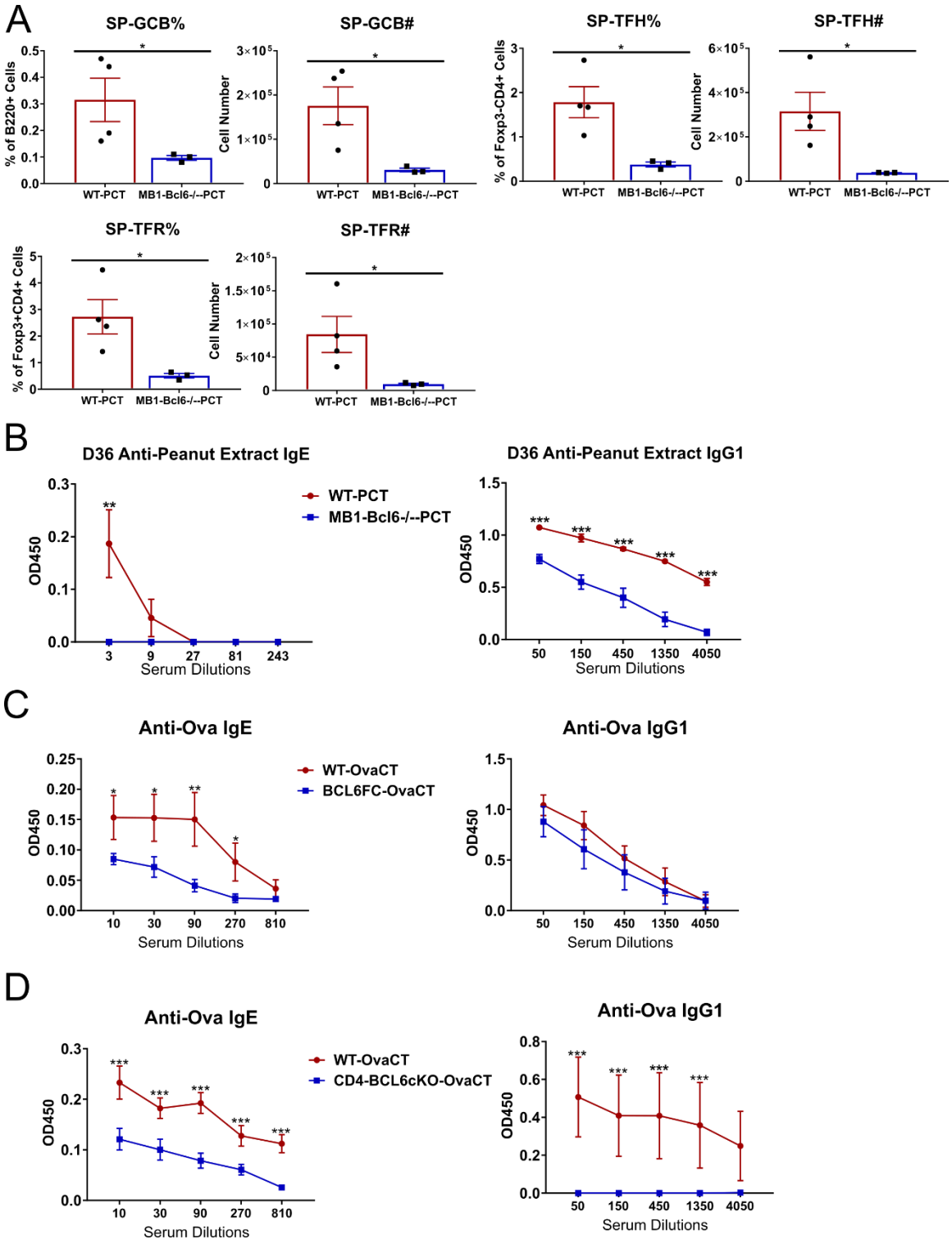

Figure 22: Block of GC B cell development leads to loss of TFH, TFR cells and peanut-specific IgE.

Figure 22: Block of GC B cell development leads to loss of TFH, TFR cells and peanut-specific IgE. (A) WT and Mb1-cre Bcl6-flox (MB1-Bcl6-/-) mice were challenged as in Figure 21 with PCT then at D36, mesenteric lymph nodes (LN) and spleens (SP) analyzed for the indicated cell populations by flow cytometry. Graphs show average % of cells as a fraction of parental cell population and total yield of cells. GCB, TFH and TFR cells analyzed as in Figure 22. (B) Peanut-specific IgE and IgG1 titers from D36 serum of WT and MB1-Bcl6-/- mice challenged with PCT. (C) Ova-specific IgE and IgG1 titers from D36 serum of WT and Bcl6FC mice challenged twice with Ovalbumin + cholera toxin. (D) Ova-specific IgE and IgG1 titers from D36 serum of WT and CD4-Bcl6cKO mice challenged twice with Ovalbumin + cholera toxin.

TFR cells are required to maintain GC responses over time

We wondered if loss of Ag-specific IgE represented a loss of IgE switching in B cells in the GCs of Bcl6FC mice. However, we did not observe a significant difference in the percentage of IgE+ GCB cells between WT and Bcl6FC mice (Fig. 23A). We then examined cells of the GC response to see if there was a defect in the GC reaction itself. As expected, TFR cells were almost completely absent in Bcl6FC mice despite a robust TFR response in WT mice with PCT challenge (Fig. 24A). Surprisingly, we observed a significant decrease in TFH cells in Bcl6FC mice after PCT challenge (Fig. 24B) and an even larger loss of GCB cells (~70% decrease) in Bcl6FC mice after PCT challenge (Fig. 24C). We examined the time course of the GC response and saw in WT mice, TFR, TFH and GCB cells all increased over time and remained high 4 weeks after the last challenge (D36; Fig. 24D). The TFH and GCB cell responses looked basically normal at the early stages in Bcl6FC mice, but, decreased at later stages of the analysis. In particular, there was a sharp decrease in GCB cells at day 36 of the response. GCB cells from WT mice continued to expand up to day 36 but in Bcl6FC mice lacking TFR cells, GCB cell growth leveled off at day 15. We confirmed these trends in a more robust PCT priming model involving 8 total PCT challenges, showing that TFR cells were still required even in a response with near saturating Ag (Fig. 23 B-D). These data indicate that in the PCT food allergy model, TFR cells are required to promote and/or maintain GC growth.

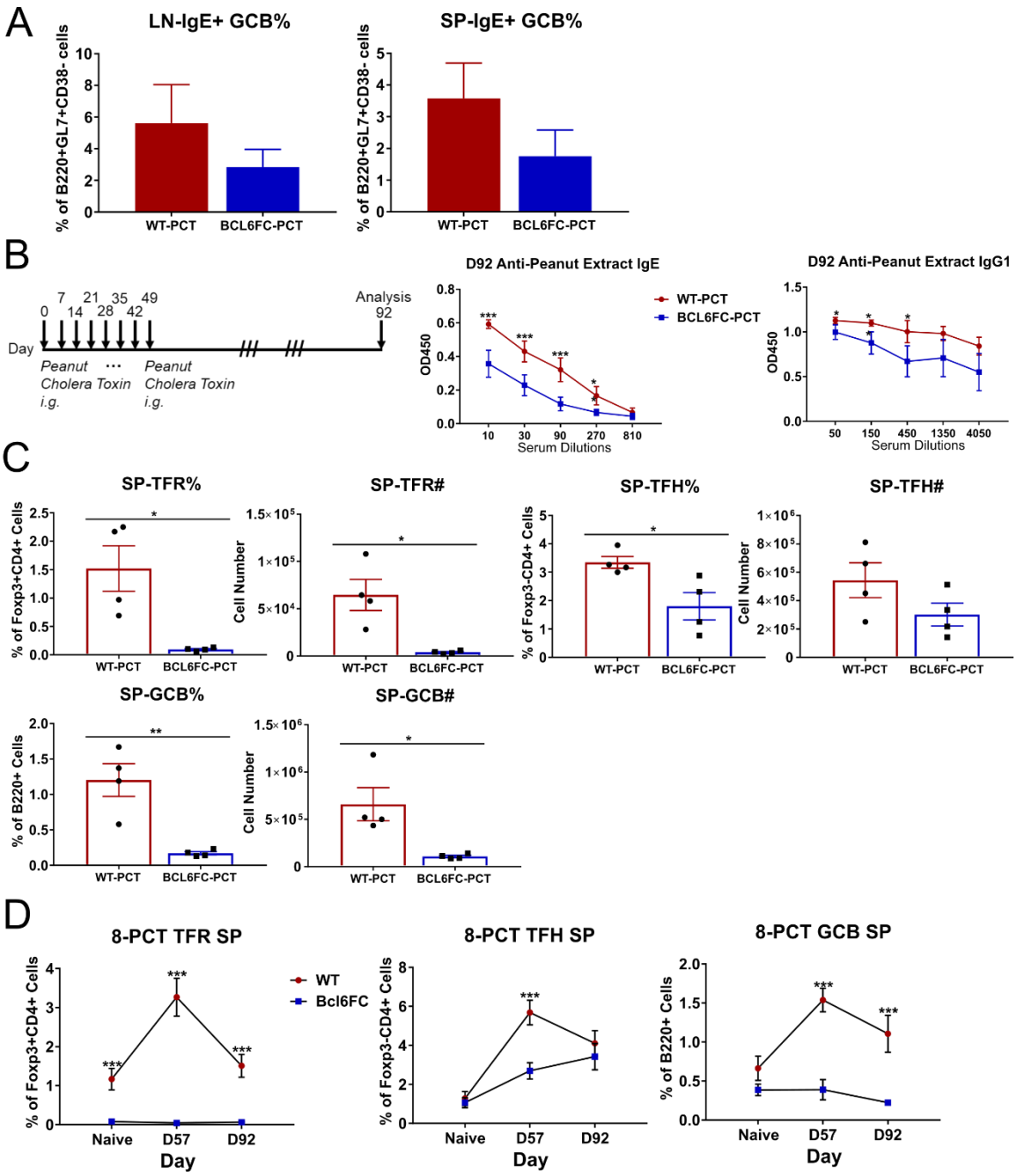

Figure 23: TFR cells are required for normal GC responses and IgE levels after 8 challenge food allergy priming.

Figure 23: TFR cells are required for normal GC responses and IgE levels after 8 challenge food allergy priming. (A) IgE+GCB cells in WT and Bcl6FC mice as a % of total GCB cells. (B) This food allergy priming uses 8 i.g. challenges of PCT, each 7 days apart, then bleeds at different time-points after challenge. shows the day 36 (D36) time-course, where immune cells and serum tested 43 days after the last challenge. Peanut-specific IgE and IgG1 titers from D92 serum of WT and Bcl6FC mice challenged 8 times with PCT. (C) WT and Mb1-cre Bcl6-flox (MB1-Bcl6-/-) mice were challenged as in part (A) with PCT then at D92, mesenteric lymph nodes (LN) and spleens (SP) analyzed for the indicated cell populations by flow cytometry. Graphs show average % of cells as a fraction of parental cell population and total yield of cells. GCB, TFH and TFR cells analyzed as in Figure 22. (D) Time-course of the TFR, TFH and GCB response after 8 PCT challenges. D15 = 7 days after challenge, D56 = 7 days after last challenge, D92 = 43 days after last challenge.

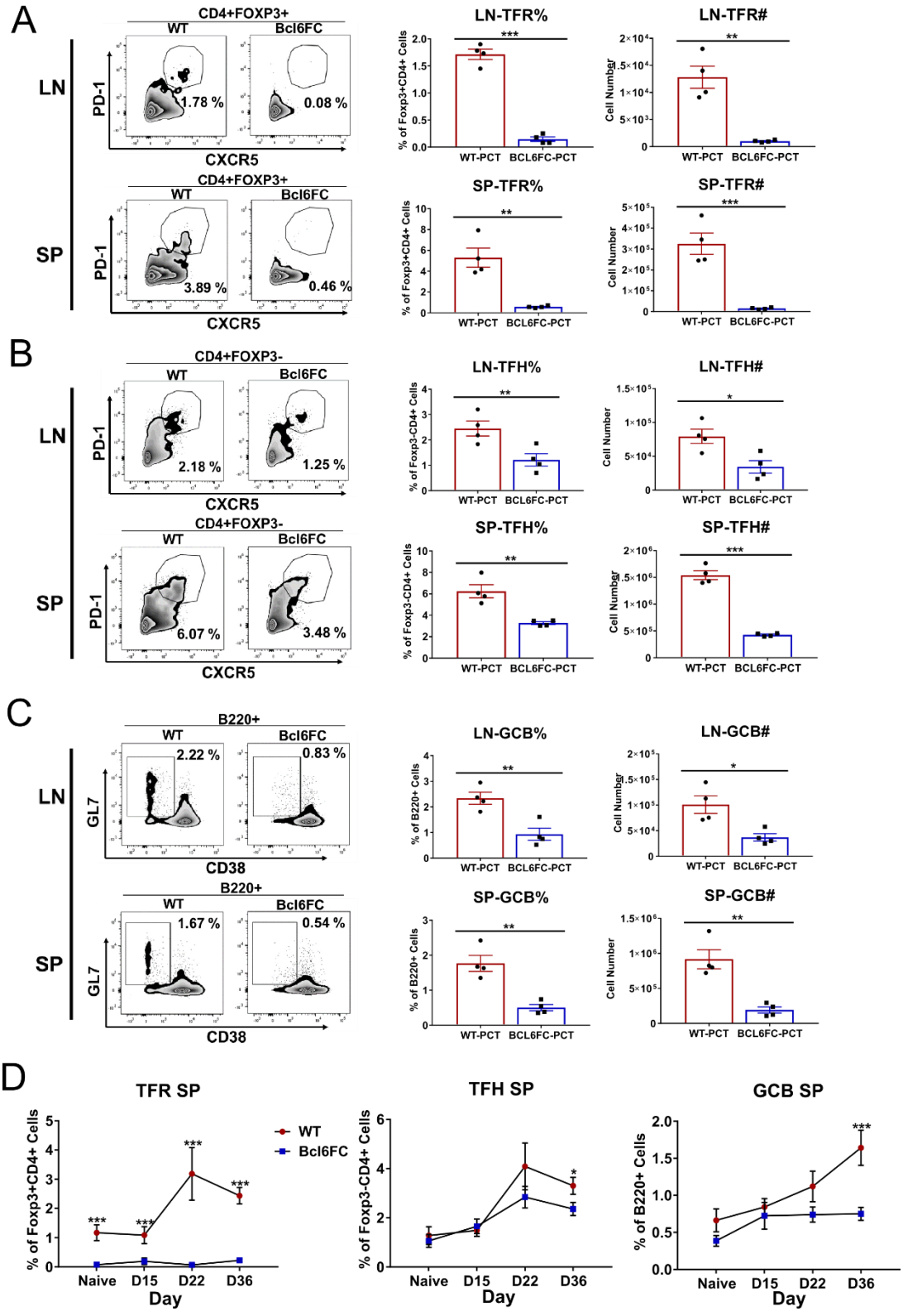

Figure 24: TFR cells are required for normal TFH and GC B cell numbers in a food allergy immune response.

Figure 24: TFR cells are required for normal TFH and GC B cell numbers in a food allergy immune response. WT and Bcl6FC mice were challenged as in Figure 21 with PCT then at D36, mesenteric lymph nodes (LN) and spleens (SP) analyzed for the indicated cell populations by flow cytometry. Representative contour dot plots for each cell staining are shown along with graphs showing average % of cells as a fraction of parental cell population and total yield of cells. (A) Analysis of CD4+Foxp3+PD-1+CXCR5+ TFR cells. TFR cells are quantitated as a percentage of Foxp3+CD4+ T cells, and absolute number per LN or SP. (B) Analysis of CD4+Foxp3- PD-1+CXCR5+ TFH cells. TFH cells are quantitated as a percentage of Foxp3- CD4+ T cells, and absolute number per LN or SP. (C) Analysis of B220+CD38- GL7+ GCB cells. GCB cells are quantitated as a percentage of B220+ cells, and absolute number per LN or SP. (D) Time-course of the TFR, TFH and GCB responses after PCT challenges. D15 = 7 days after challenge, D22 = 14 days after challenge, D36 = 28 days after challenge. P values were calculated by t test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. N = 4 - 6 mice, and each experiment was repeated 2 to 8 times.

Treg cells are required for antigen-specific IgE production in the food allergy model

We next wondered whether our results with Bcl6FC mice were unique to this mouse model of TFR deficiency. We therefore obtained Foxp3-diptheria toxin receptor (Foxp3-DTR) mice where Foxp3+ Treg cells can be depleted by injection of diphtheria toxin (DT), causing loss of TFR cells (49, 137), and tested them in the PCT model as shown in Fig. 25A. Similar to our results with Bcl6FC mice, deletion of total Tregs led to a dramatic loss of peanut-specific IgE and a significant but not complete loss in peanut-specific IgG1 (Fig. 25B). We then confirmed that the DT treatment led to essentially complete deletion of Tregs at an early stage of the response in the Foxp3-DTR mice (Fig. 25C, D). These data support the idea that TFR cells are required for producing Ag-specific IgE.

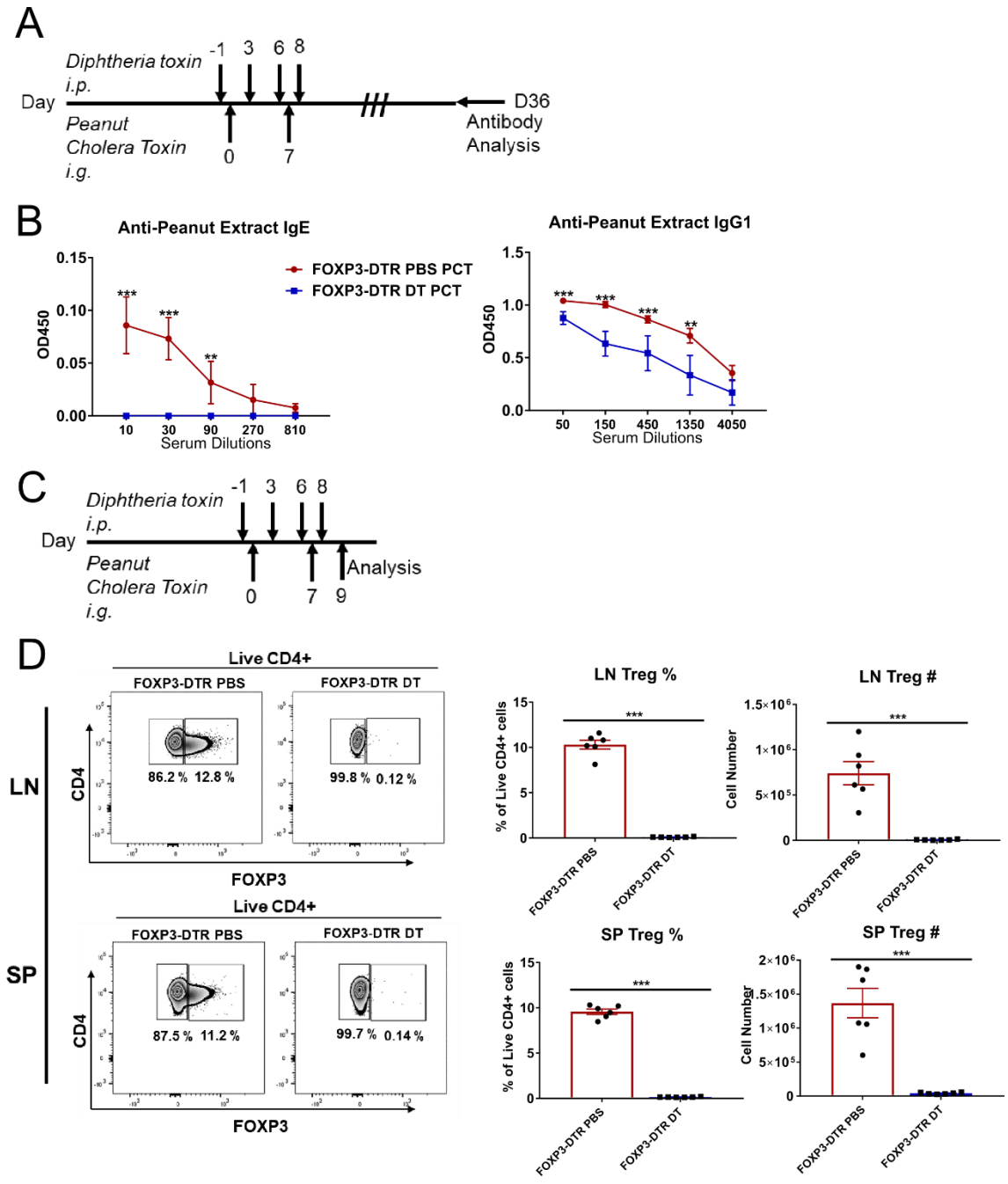

Figure 25: Total Treg and TFR cells are required for antigen-specific IgE and IgG1 in a food allergy response.

Figure. 25. Total Treg and TFR cells are required for antigen-specific IgE and IgG1 in a food allergy response. (A) Foxp3-DTR mice were treated with diphtheria toxin (DT) as indicated to deplete Treg cells, or given PBS as a control, challenged with PCT as indicated, and bled for serum peanut-specific IgE and IgG1 Ab on D36 (B). (C) Foxp3-DTR mice were treated with DT or given PBS as a control, challenged with PCT at days 0 and 7 as indicated, and then at day 9, draining mesenteric lymph node (LN) and spleen (SP) taken for analysis of Foxp3+ Treg cells by flow cytometry (D). Representative contour dot plots for Treg staining are shown along with graphs showing average % of Treg cells as a fraction of CD4 T cells and total yield of Treg cells. P values were calculated by t test or ANOVA where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. N = 5 - 8 mice, and each experiment was repeated 2 times.

TFR cells actively promote antigen-specific IgE

Although our experiments showed that TFR cells were necessary for peanut-specific IgE responses in the PCT model, our data did not indicate if TFR cells were simply required at some minimal level in the GC response or if TFR cells actively promoted peanut-specific GCB cell responses. We therefore used a mouse line previously shown to develop augmented TFR responses due to a specific deletion of Pten expression in Treg cells (Foxp3-cre Pten-flox or PtenFC mice (174)) to test this hypothesis. We challenged PtenFC mice with PCT and analyzed the resulting immune response. As shown in Fig. 26A, peanut-specific IgE was strongly increased in PtenFC mice compared to WT mice, while peanut-specific IgG1 production was not affected. We confirmed TFR cells were significantly augmented in the PCT-challenged PtenFC mice (Fig. 26B), and observed that this increase was associated with an increase in both TFH and GCB cells (26C, D; Fig. 27). These data challenge the notion that TFR cells act as suppressors of the GC response, as is typically proposed for TFR function. Instead our data show that TFR cells crucially drive GC responses in this food allergy model, and this idea is supported by a linear correlation analysis between numbers of TFR cells and other cells in the GC (Fig. 26E). As expected, numbers of TFH and GCB cells were tightly correlated, but TFR cells also positively correlated with both GCB and TFH cells. This finding, coupled with the weak GCB cell response in Bcl6FC mice suggests that TFR cells are active helper cells of the Ab response in the PCT food allergy model.

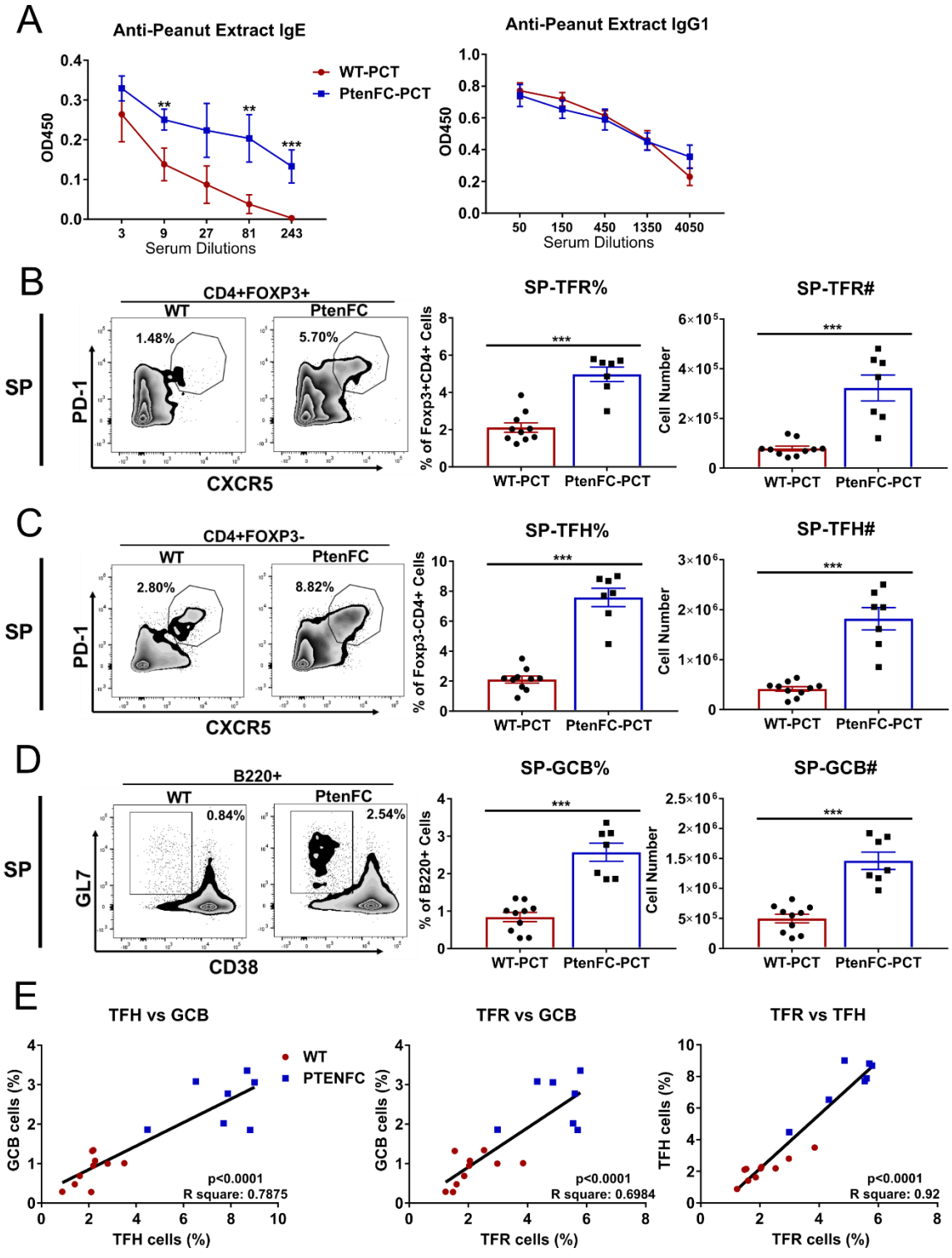

Figure 26: Augmented TFR cell development promotes higher IgE and correlates with increased GC responses after food allergy challenge.

Figure 26: Augmented TFR cell development promotes higher IgE and correlates with increased GC responses after food allergy challenge. WT and Foxp3-cre Pten-flox (PtenFC) mice were challenged as in Figure 21 with PCT. At D36 of the challenge system, (A) serum was tested for peanut-specific Abs, and (B-D) spleens (SP) were analyzed for TFR cells, TFH cells and GCB cells by flow cytometry as in Figure 22. Representative contour dot plots for each cell staining are shown along with graphs showing average % of cells as a fraction of parental cell population and total yield of cells. (E) Linear correlation analysis between TFH cell % and GCB cell %, between TFR cell % and GCB cell % and between TFR cell % and TFH cell %, using data combined from WT and PtenFC mice. P values were calculated by t test or ANOVA where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. R square values in (E) were calculated by Prism GraphPad software. N = 4 mice. Each experiment was repeated 2 times.

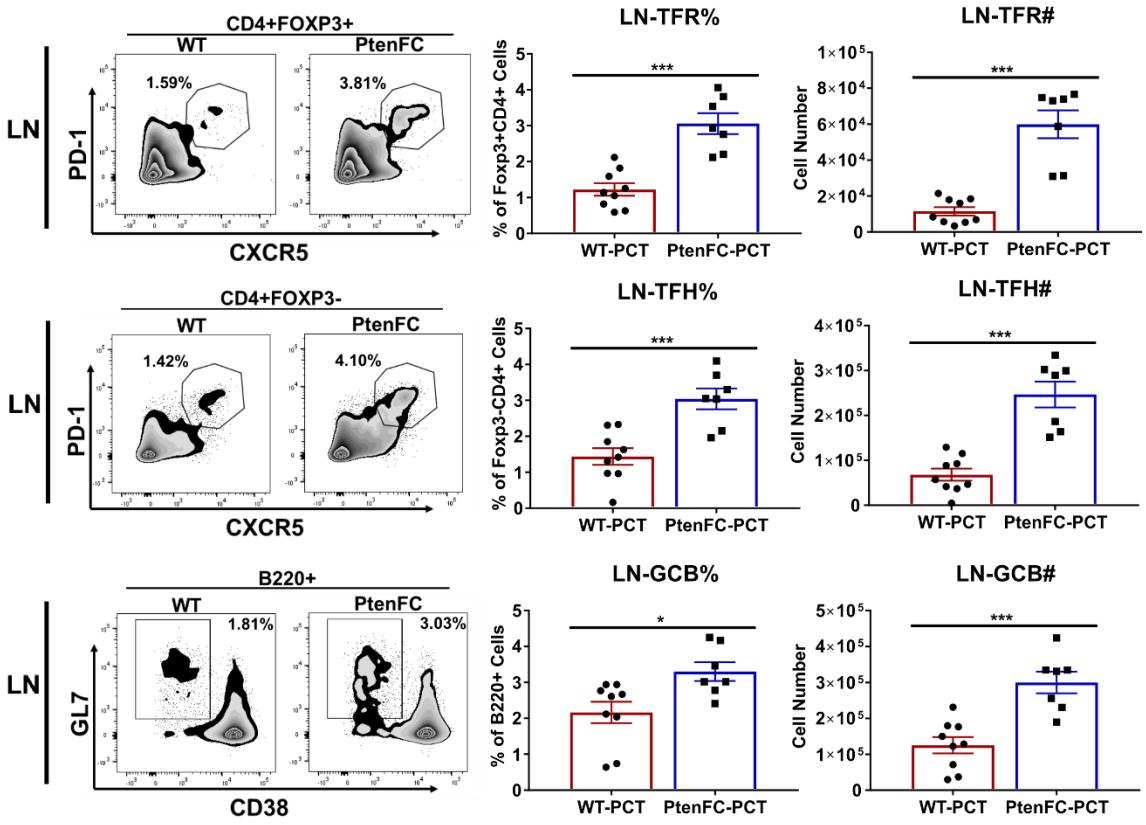

Figure 27: Augmented TFR, TFH and GC B cells responses in the lymph node of Pten conditional knockout mice after food challenge. WT and Foxp3-cre Pten-flox (PtenFC) mice were challenged as in Figure 21 with PCT. At D36 of the challenge system, LN were analyzed for TFR cells, TFH cells and GCB cells by flow cytometry as in Figure 22. Representative contour dot plots for each cell staining are shown along with graphs showing average % of cells as a fraction of parental cell population and total yield of cells. P values were calculated by t test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. R square values in (E) were calculated by Prism GraphPad software. N = 4 mice. Each experiment was repeated 2 times.

Blimp1-controlled IL-10 is key for IgE responses

We next tested TFR cell function in the PCT food allergy model using Blimp1FC mice, where Blimp1 is specifically deleted in Tregs cells by Foxp3-cre. Since Blimp1 is an antagonistic transcriptional regulator of the follicular T cell differentiation and Blimp1 represses the master follicular T cell transcription factor Bcl6 (51, 82), Blimp1FC mice are predicted to have an augmented TFR cell response, due to higher Bcl6 expression. Indeed, we found markedly increased TFR cells in Blimp1FC mice when are challenged with PCT (Fig. 28A), and consistent with our other findings in this model, TFH and GCB cells were also elevated (Fig. 28 B, C). However, when we tested for peanut-specific IgE and IgG1, the levels made by Blimp1FC mice were comparable to WT levels (Fig. 28D). These results show that the increased TFR cells in Blimp1FC mice did not boost Ab production, which contrasts to what we saw with PtenFC mice with increased TFR cells. Since Blimp1 is known to positively control IL-10 expression in Treg cells (196-198), and TFR cells produce IL-10 that helps promote GC responses (68), we wondered if loss of IL-10 production from Blimp1-deficient TFR cells was causing the lack of IgE increase after PCT challenge. We used ELISA to test IL-10 expression and found about a 6-fold decrease in IL-10 secretion from Blimp1FC Tregs (Fig. 28E). To test if other Treg or TFR cell genes were altered in Blimp1FC TFR cells besides IL-10, we used RNAseq to profile gene expression in FACS sorted TFR cells from WT and Blimp1FC mice. Overall, 198 (1.6%) of the sequenced genes were differentially expressed between WT and Blimp1FC TFR cells (data not shown). When we analyzed a panel of key Treg and TFR genes

profiled by RNAseq, we saw that only IL-10 was strongly affected by loss of Blimp1, with a 7-fold decrease in Il10 RNA (Fig. 28F). These data indicate that even though TFR cells are produced at higher levels in Blimp1FC mice, they are defective at promoting higher peanut-specific IgE in the PCT model, likely because of a deficiency in IL-10 production.

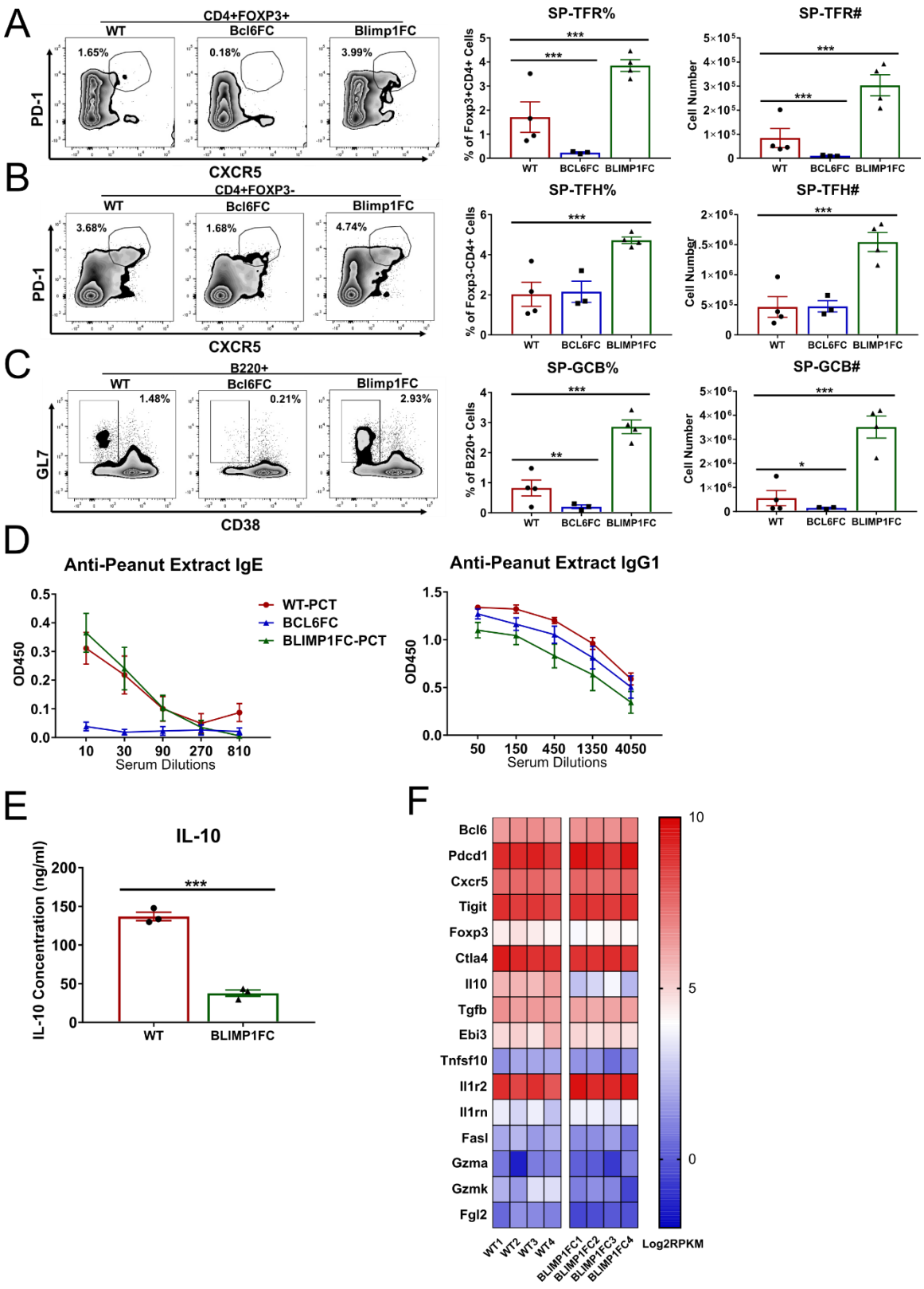

Figure 28: Blimp1-dependent IL-10 produced by TFR cells helps promote IgE responses in food allergy.

Figure 28: Blimp1-dependent IL-10 produced by TFR cells helps promote IgE responses in food allergy. (A-D) WT, Bcl6FC and Foxp3-cre Blimp1-flox (Blimp1FC) mice were challenged as in Figure 21 with PCT. At D36 of the challenge system, spleens (SP) were analyzed by flow cytometry as in Figure 22 for (A) TFR cells, (B) TFH cells, (C) GCB cells, and (D) serum was tested for peanut-specific Abs. Representative contour dot plots for each cell staining are shown along with graphs showing average % of cells as a fraction of parental cell population and total yield of cells. (E) IL-10 ELISA data for 72 hr supernatants from CD3+CD28 stimulated Tregs from WT, Bcl6FC and Blimp1FC mice. P values were calculated by t test or ANOVA where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. N = 5 mice, and each experiment was repeated 2 times. (F) Heat map showing RNA transcript levels of key follicular T cell and Treg cell genes in TFR cells from WT and Blimp1FC mice. Transcript levels were determined by RNAseq. TFR cells were isolated by FACS from PCT challenged mice (N= 4 of each strain). Color scale representing absolute RNA levels is shown on the right as Log2RPKM values.

IL-10 is critical for antigen-specific IgE responses

To further clarify the role of IL-10 in regulating peanut-specific IgE in our food allergy model, we used Mb1-cre IL-10Ra-flox (MB1-Il10ra-/-) mice, where the IL-10 receptor alpha gene is deleted specifically in B cells and thus the B cells cannot respond to IL-10 signals. These mice were challenged with PCT and tested for GC and peanut-specific Ab responses. As shown in Fig. 29A-B, loss of IL-10 signaling in B cells led to significantly decreased GCB cell responses and a complete loss of peanut-specific IgE, while leaving the peanut-specific IgG1 response intact. We also tested germline IL-10-/- mice with PCT challenge and found significant decreases in peanut-specific IgE and IgG1 (Fig. 29C), again supporting a key role for IL-10 in controlling the IgE response to a food allergen. We then wondered if we could block IgE production in WT mice with anti-IL-10R Ab after PCT challenge, an approach with therapeutic potential. As shown in Fig. 29D-E, repeated doses of anti-IL10R Ab in WT mice after PCT challenge led to a dramatic loss of peanut-specific IgE by day 29 with only a small drop in peanut-specific IgG1 at day 29. Significantly, anti-IL10R Ab treatment also strongly inhibited anaphylaxis after peanut challenge (Fig. 29F), mirroring the loss of IgE production. We also tested the role of IL-10 signaling on CD4 T cells with CD4-cre IL10Ra-flox mice and the PCT challenge system, however there was little difference in anti-peanut IgE and IgG1 and the GC response in these mice (Fig. 30). These data support the idea that IL-10 acts on GCB cells to promote the IgE response. To further investigate the effect of IL-10 acting on GCB cells, we examined the light zone and dark zone composition of GCB cells in MB1-Il10ra-/-

mice, and found a large shift to light zone GCB cells in these mice (Fig. 31A). These data are consistent with recent findings that IL-10 promotes entry of the GCB cell into the dark zone compartment of the GZ (68), and thus without IL-10 signaling, GCB cells tend to accumulate in the light zone. We further found an extreme shift of GC B cells to the light zone in Bcl6FC mice (Fig. 31B), which is consistent with a loss of TFR cell derived IL-10 in Bcl6FC mice. Lastly, we observed a 2-3 folds increase in dead GCB cells in Bcl6FC mice (Fig. 31C), which is likely related to loss of IL-10 signaling and can explain the loss of GCB cells in these mice.

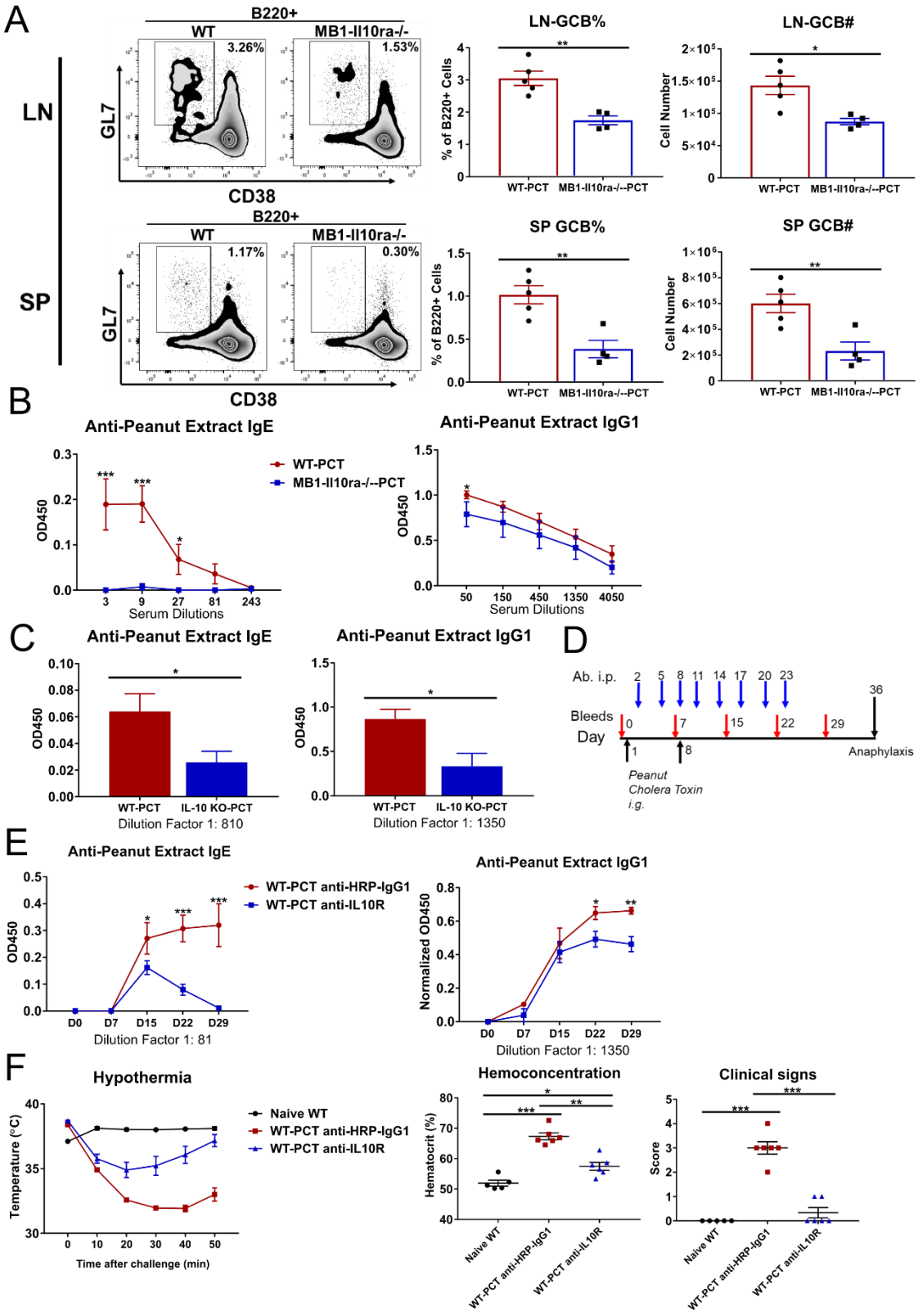

Figure 29: IL-10 promotes GC B cell levels and peanut-specific IgE, and therapeutic blockade of IL-10 during food allergy challenge leads to loss of IgE.

Figure 29: IL-10 promotes GC B cell levels and peanut-specific IgE, and therapeutic blockade of IL-10 during food allergy challenge leads to loss of IgE. (A) WT and Mb1-cre IL-10Ra-flox (MB1-Il10ra-/-) mice were challenged with PCT and at D36, and GCB cells from LN and SP were stained and analyzed by flow cytometry. Representative contour dot plots of GCB cell staining are shown along with graphs showing average % of GCB cells and total yield of cells. (B) Peanut-specific IgE and IgG1 titers from D36 serum of WT and MB1-Il10ra-/- mice challenged with PCT. (C) Peanut-specific IgE and IgG1 levels from D36 serum of WT and Il10-/- (IL-10 KO) mice challenged with PCT. (D) Scheme for block of IL-10 receptor during PCT challenge in female C57Bl/6 WT mice. Numbers indicate specific days for i.p. anti-IL10R Ab treatment, i.g. PCT gavage, blood sampling and anaphylaxis. Control mice received anti-HRP-IgG1 Ab. (E) Peanut-specific IgE and IgG1 titers from serum of control and anti-IL10R mice treated as described in (D) at the indicated timepoints. (F) Anaphylaxis response of control and anti-IL10R mice treated as described in (D). Anaphylaxis analysis was performed as in Figure 21. P values were calculated by t test or ANOVA where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. N = 4 - 6 mice, and each experiment was repeated 2 times.

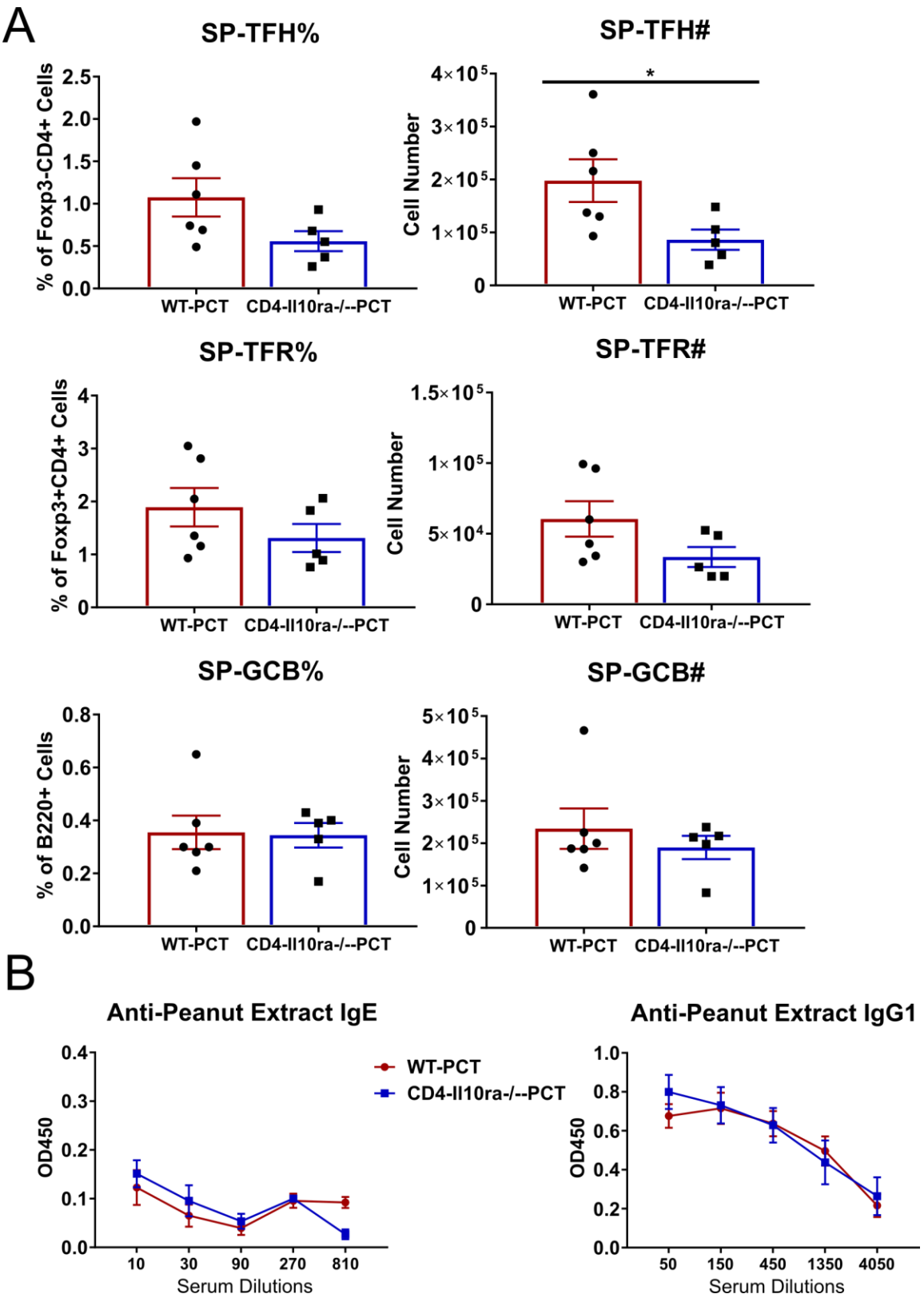

Figure 30: IL-10 signaling on T cells does not affect food allergy GC response and IgE production.

Figure 30: IL-10 signaling on T cells does not affect food allergy GC response and IgE production. (A) WT and CD4-cre IL10ra-flox (CD4-Il10ra-/-) mice were challenged as in Figure 21 with PCT. At D36 of the challenge system, SP were analyzed for TFR cells, TFH cells and GCB cells by flow cytometry as in Figure 22. Graphs show average % of cells as a fraction of parental cell population and total yield of cells. (B) Peanut-specific IgE and IgG1 titers from D36 serum of WT and CD4-Il10ra-/- mice challenged with PCT. P values were calculated by t test or ANOVA where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. N = 6 mice. Each experiment was repeated 2 times.

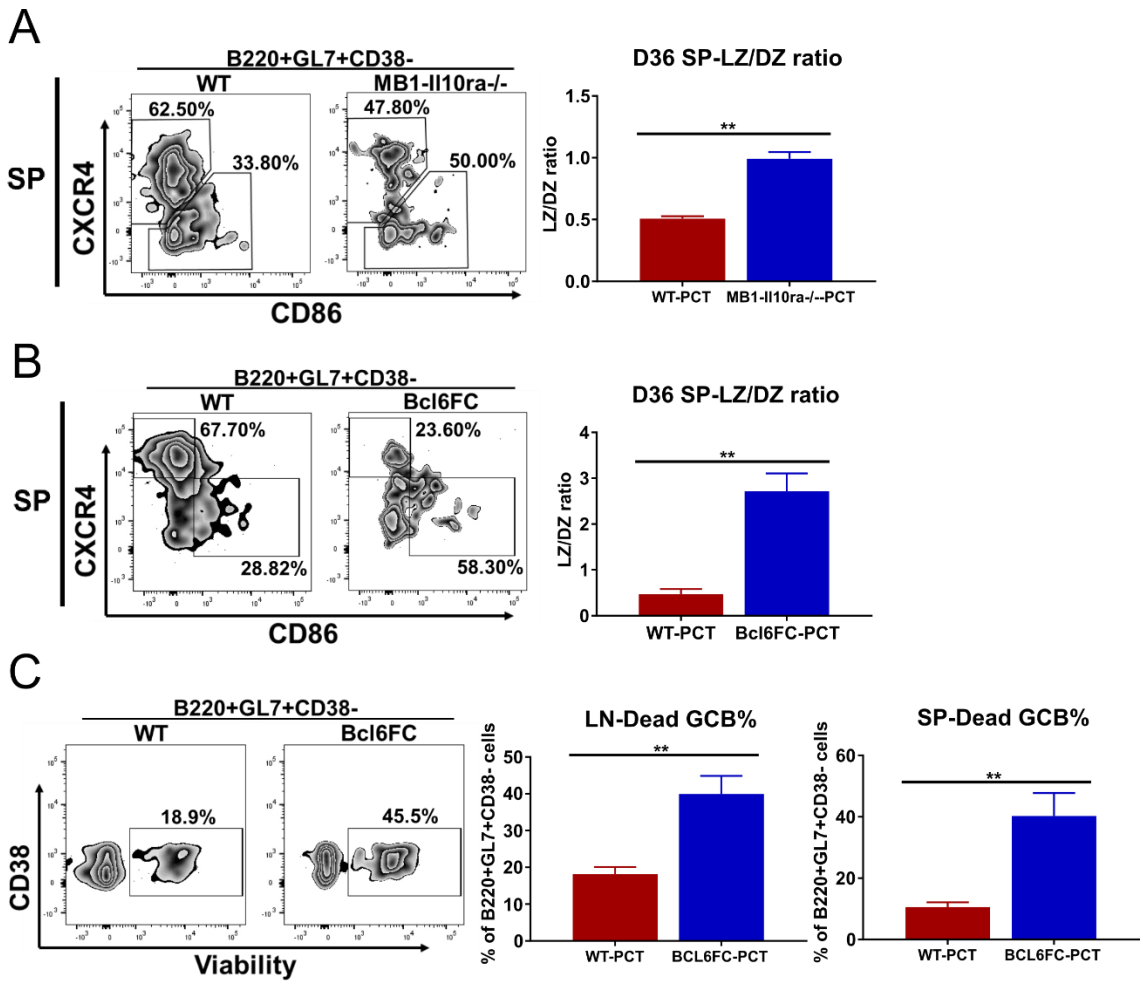

Figure 31: Altered GCB cell cycling and increased apoptosis in the absence of TFR cells. (A) WT and Mb1-cre IL-10Ra-flox (MB1-Il10ra-/-) mice were challenged with PCT and at D36, and GCB cells from SP were stained and analyzed by flow cytometry for light zone (LZ; CXCR4) and dark zone (DZ; CD86) marker expression. Representative contour dot plots of GCB DZ/LZ cell staining are shown along with graphs showing average ratios of GCB LZ to GCB DZ cells. (B) WT and Bcl6FC mice were challenged with PCT and at D36, and GCB cells from SP were stained and analyzed by flow cytometry for LZ and DZ marker expression as in part A. Representative contour dot plots of GCB DZ/LZ cell staining are shown along with graphs showing average ratios of GCB LZ to GCB DZ cells. (C) WT and Bcl6FC mice were challenged with PCT and at D36, and GCB cells from SP were stained and analyzed by flow cytometry for viability using eBioscience™ Fixable Viability Dye. Representative viability stains are shown along with graphs showing average GCB cell death. P values were calculated by t test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. N = 4 - 5 mice, and each experiment was repeated 2 times.

PART IV: Follicular Regulatory T Cells Inhibit the Development of Aberrant Granzyme B Expressing Follicular Helper T Cells.

T follicular regulatory (TFR) cells can have both suppressive and helper roles in the germinal center (GC). Using a peanut food allergy model, we found GC B cells and peanut-specific IgE were significantly decreased in TFR-deficient mice, whereas T follicular helper (TFH) cell number was not affected. Using gene profiling, we found that TFH cells from TFR-deficient mice showed strong up-regulation of granzyme B (*Gzmb*) and other effector CD8 T cell genes. This aberrant cytotoxic T cell gene profile was strongly associated with Stat4 activity but was not associated with a canonical Th1 profile. We detected Gzmb+ and Eomesodermin+ TFH cells and a higher rate of apoptotic GC B cells in the absence of TFR cells. Our data show that TFR cells can positively regulate the GC response by repressing abnormal TFH cell differentiation, which leads to increased GC B cell numbers and increased Ag-specific Ab.

TFR cells promote stronger GC B cell and IgE responses

To study Ag-specific Ab responses, we used a classic model of food allergy induced by i.g. challenge with PCT (177, 180, 199). In this model, high levels of peanut-specific IgE are induced and maintained in circulation for weeks after immunization in normal mice. To test the role of TFR cells, we used the Bcl6FC TFR cell-deficient mouse model (109), mice where Blimp1 was deleted in Foxp3+ cells (Blimp1FC) leading to higher TFR cells and mice with deletion of both Bcl6

and Blimp1 in Foxp3+ cells (DKO). All mouse strains were healthy with no obvious disease by week 6-10. As shown in Fig. 32A, TFR cells were largely ablated in Bcl6FC and DKO mice but were increased about 2-fold over WT levels in Blimp1FC mice. These data show dominant role for Bcl6 in TFR cell development. Loss of TFR cells did not affect TFH cell numbers, however there was a marked increase in both Blimp1FC and DKO TFH cells (Fig. 32B). Blimp1 is required for IL-10 expression by regulatory T cells (Tregs; (200, 201)). TFR cell levels strongly affected GCB cell levels, showing a strong positive correlation in Bcl6FC, Blimp1FC and DKO strains (Fig. 32C, Fig. 33). These data show that TFR cells play a helper role for GCB cell responses. Indeed, we observed a 5-6 folds lower GC B cells to TFH cells in TFR cell deficient mice compared to TFR sufficient mice, indicating the TFH cells are less efficient helpers in the absence of TFR cells (Fig. 32 D, E). Analysis of peanut-specific IgE titers revealed that TFR cells are required for sustained IgE responses in this model (Fig. 32F). Overall, these findings support the idea that TFR cells positively regulate the GC B cell population and the IgE response.

TFR cells inhibit the development of aberrant cytotoxic gene expressing TFH cells

To better understand how TFR cells were influencing the ability of TFH cells to help GCB cells in our model, we used RNAseq to profile gene expression in

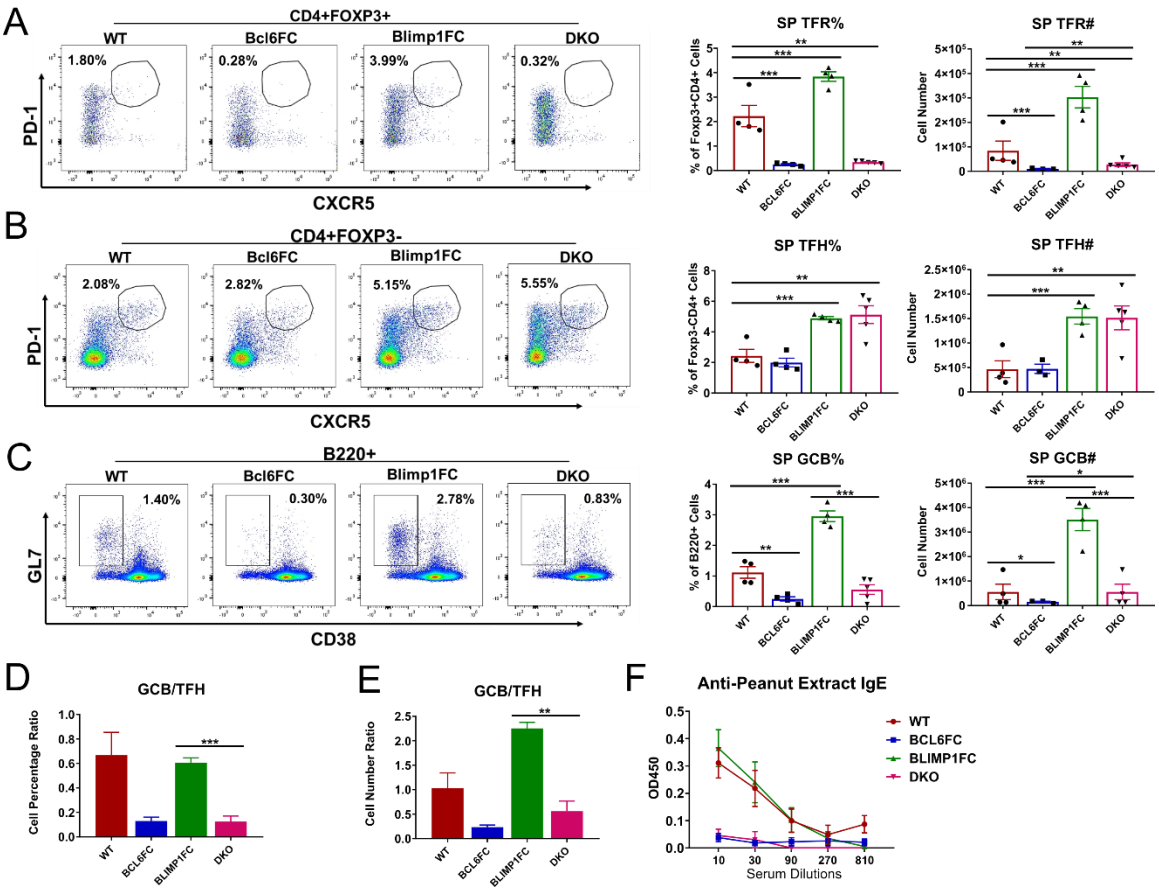

Figure 32: TFR cells are required for proper GC B cell numbers in a food allergy immune response. WT, Bcl6FC, Blimp1FC and DKO mice were orally immunized twice with peanut protein + cholera toxin (PCT). 4 weeks after the last PCT immunization (day 36), spleens (SP) were analyzed for the indicated cell populations by flow cytometry. Representative contour dot plots for each cell staining are shown along with graphs showing average % of cells as a fraction of parental cell population and total yield of cells. (A) Analysis of CD4+FOXP3+PD-1+CXCR5+ TFR cells. Average TFR cells per group are quantitated as a percentage of FOXP3+CD4+ T cells, and absolute number. (B) Analysis of CD4+FOXP3- PD-1+CXCR5+ TFH cells. Average TFH cells are quantitated as a percentage of FOXP3- CD4+ T cells, and absolute number. (C) Analysis of B220+CD38- GL7+ GCB cells. Average GCB cells per group graphed as a percentage of B220+ cells and as absolute number. (D-E) Ratio of GC B cells to TFH cells from data in (A-C). (F) Titers of peanut-specific IgE by ELISA at day 36. P values were calculated by t test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. N = 4 - 6 mice; experiment was repeated twice with similar results.

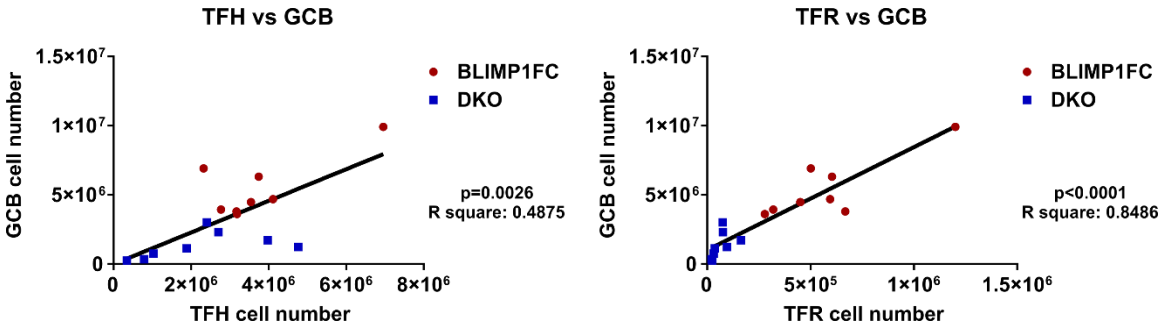

Figure 33: Linear regressions between TFH and GCB cells (left plot), TFR and GCB cells (right plot) of Blimp1FC and DKO.

TFH cells from PCT-challenged WT, Bcl6FC, Blimp1FC and DKO mice (Fig. 34, Fig. 35). TFH gene expression was strongly affected by loss of TFR cells, leading to several hundred up-regulated and down-regulated DEGs for both Bcl6FC and DKO TFH cells, respectively (Fig 34A). Notably, Granzyme B (*Gzmb*) was strongly elevated in both Bcl6FC and DKO TFH cells (Fig 34A). Despite the large number of DEGs, the expression of key TFH genes remained intact (Fig. 34B). To better understand the nature of the DEGs in Bcl6FC and DKO TFH cells, we compared up-regulated DEGs from our dataset with published gene expression datasets. We found a highly significant enrichment of genes that were up-regulated in effector CD8 T cells and controlled by Stat4 in Th1 cells within the up-regulated DEGs from Bcl6 and DKO TFH cells (Fig. 34C, D). We then selected 23 genes up-regulated during CD8 effector T cell differentiation (Fig. 34E), which included genes for the cytotoxic proteins Gzmb and Perforin 1 (*Prf1*). About 75% of these 23 genes were dependent on Stat4 for expression and all 23 genes were robustly up-regulated in

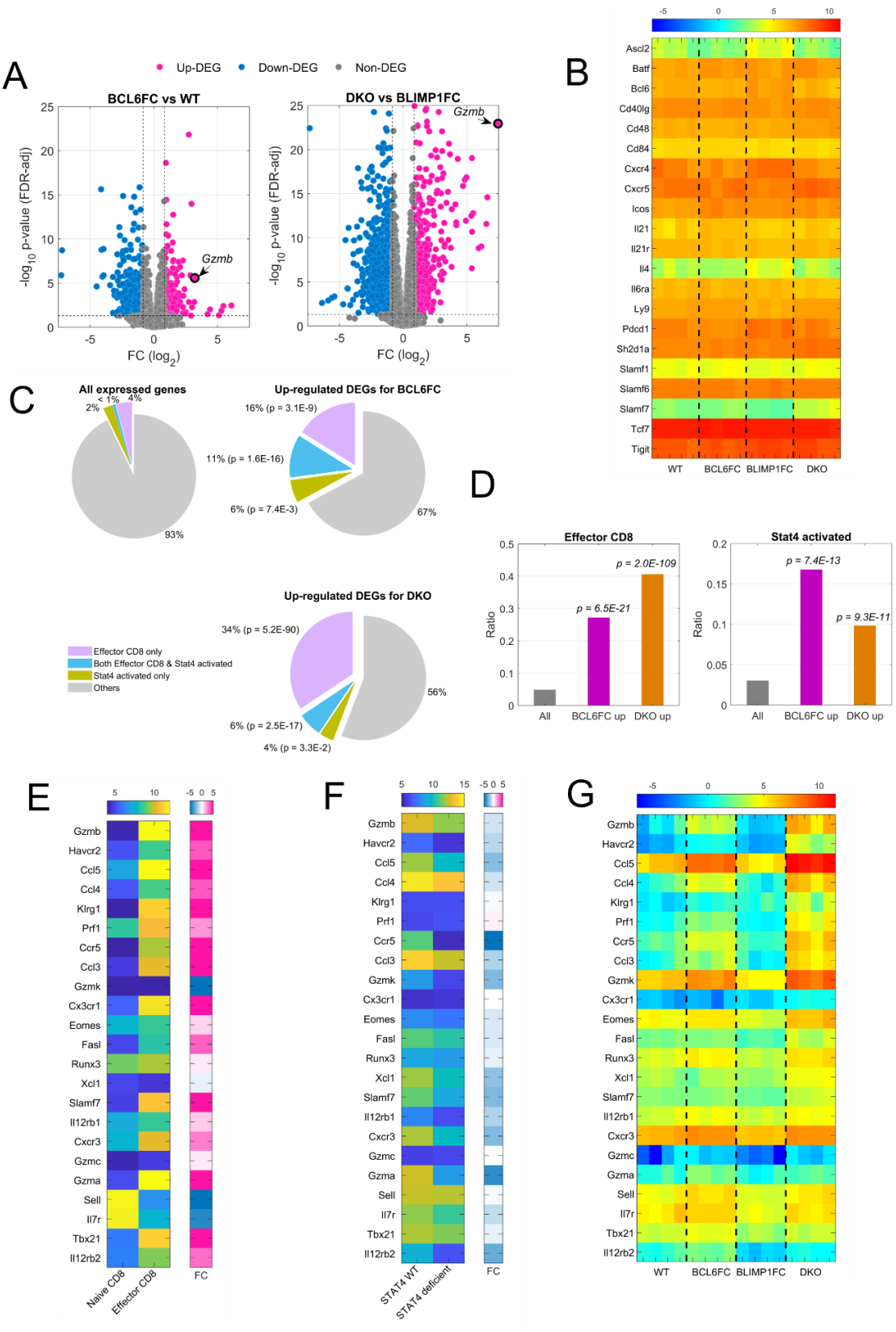

Figure 34: TFR cells repress a cytotoxic gene program in TFH cells.

Figure 34: TFR cells repress a cytotoxic gene program in TFH cells. WT, Bcl6FC, Blimp1FC and DKO mice were orally immunized twice with PCT (N = 4). 4 weeks after the last immunization, TFH cells were purified from spleens by FACS. RNA was isolated from the TFH cells directly after sorting and subjected to RNAseq. (A) Volcano plots showing DEGs for Bcl6FC versus WT (155 genes up, 410 genes down) and DKO versus Blimp1FC (517 genes up, 1041 down). Blue are down-regulated genes and purple are up-regulated genes, using FDR <0.05 and fold-change >1.8 (linear). *Gzmb* is specifically marked in both plots. (B) Heat map showing expression of hallmark TFH genes assessed with RNAseq. Color scale shows log2 RPKM level of absolute expression of genes sorted alphabetically. (C-D) Enrichment of DEGs in published datasets for genes associated with effector CD8 differentiation (GSE36168) (202) and Stat4 regulation in Th1 cells (GSE22105) (24), for Bcl6FC versus WT and DKO versus Blimp1FC comparisons, analyzing up-regulated DEGs and fraction of these genes within all expressed genes and probability (p values) for the enrichment. (C) pie charts showing unique and overlapping CD8 effector and Stat4 activated genes, (D) shows graphs of enrichment of only CD8 effector and Stat4 activated genes. (E-G) A set of 23 hallmark genes was chosen for strong association with CD8 effector T cell differentiation, and used to create blue to yellow/red heat maps based on Log2RPKM gene expression for (E) naïve and effector CD8 T cells (GSE36168), (F) WT and Stat4-/- Th1 cells (GSE22105) and (G) TFH cells from WT, Bcl6FC, Blimp1FC and DKO mice. In (E-F), fold-change (FC) for the paired sets of genes is shown by a single column heat map.

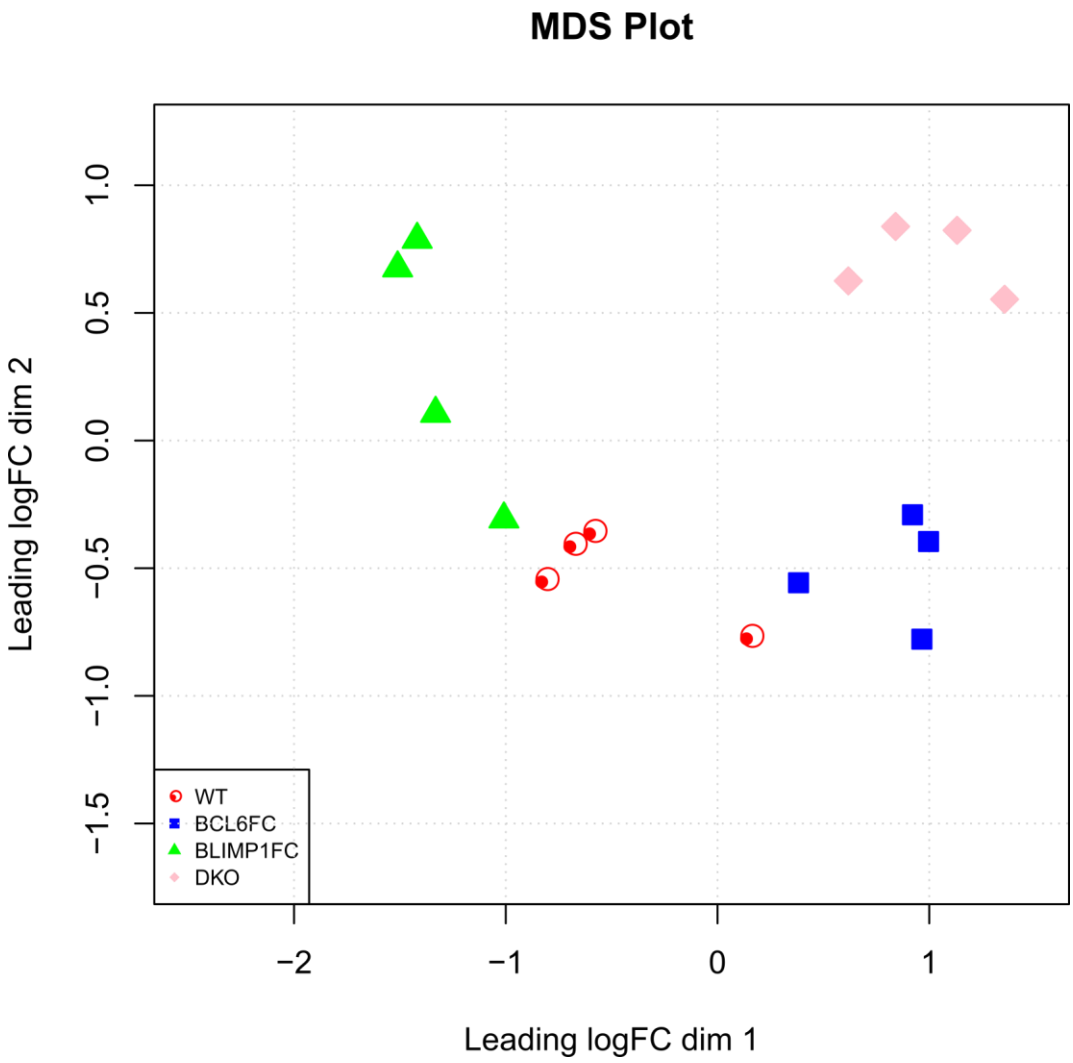

Figure 35: MDS plot of TFH cells from RNAseq analysis. (Figure prepared by Dr. Jun Wan of IU). Bcl6FC, Blimp1FC and DKO mice were orally immunized twice with PCT (N = 4). 4 weeks after the last immunization, TFH cells were purified from spleens by FACS. RNA was isolated from the TFH cells directly after sorting and subjected to RNAseq.

Bcl6FC and DKO TFH cells compared to WT TFH cells (Fig. 34F, G). The 23 CD8 cytotoxic effector genes were up-regulated to a much higher degree in DKO TFH cells than in Bcl6FC TFH, indicating a unique regulatory environment in those mice beyond loss of TFR cells. However, the expression of the 23 CD8 effector genes was nearly identical between WT and Blimp1FC TFH cells, also suggest augmented TFR cells in Blimp1FC do not affect TFH gene profile, showing that the highly up-regulated CD8 effector gene program in the DKO TFH cells was not simply due to loss of Blimp1 in Treg cells. Although the CD8 effector T cell gene profile seen in Bcl6FC and DKO TFH cells was significantly dependent on Stat4, these cells did not have a classical Th1 gene profile, as *Ifng* expression was not increased in these TFH cells and there was no clear Tbet (*Tbx21*) function (Fig. 36A, B). Although we observed this CD8 effector T cell gene profile in TFR mutant mice, we wondered if this cytotoxic-like profile might appear in a virus infection system. We therefore used a published RNAseq dataset of TFH cells from SIV-infected Macaques, and observed that there was a highly significant increase in expression of the 23 CD8 effector genes in SIV+ versus SIV- TFH cells (Fig. 36C). These data indicate that the aberrant TFH cell phenotype that develops in the absence of TFR cells can develop in a normal GC response after virus infection.

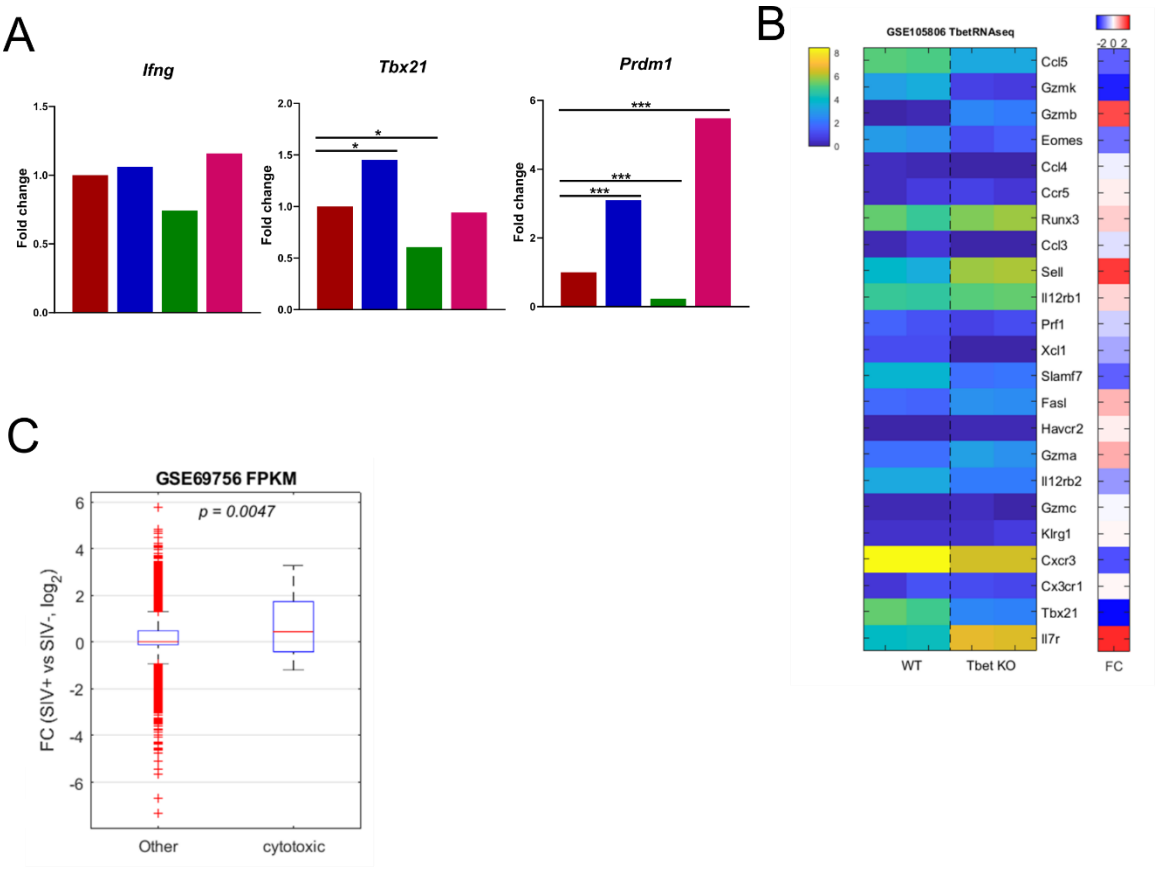

Figure 36: Expression of Th1 genes in TFH cells, Tbet regulation of cytotoxic genes, and cytotoxic TFH genes up-regulated after SIV infection. (A) gene expression plots for Ifng, Tbx21 (Tbet) and Prdm1 (Blimp1) taken from the RNA-seq data, where WT (red) TFH cells levels are set to 1 and fold changes are shown for Bcl6FC (blue), Blimp1FC (green) and DKO (magenta) TFH cells. Figure color codes correspond to Figure 32D. (B) Heat map of the gene set of 23 CD8 cytotoxic T cell genes described in Figure 34D-F, versus published gene expression data for WT and Tbet KO T cells (GSE105806). (C) Comparison of fold-changes in genes from TFH cells from SIV+ infected Macaques over uninfected TFH cells from un-infected Macaques (published RNA-seq data GSE69756). The 23 CD8 cytotoxic T cell genes described in Figure 34D-F show a small but statistically significant increase in SIV+ TFH expressed genes over SIV- TFH expressed genes, compared to all other genes expressed in the TFH cells.

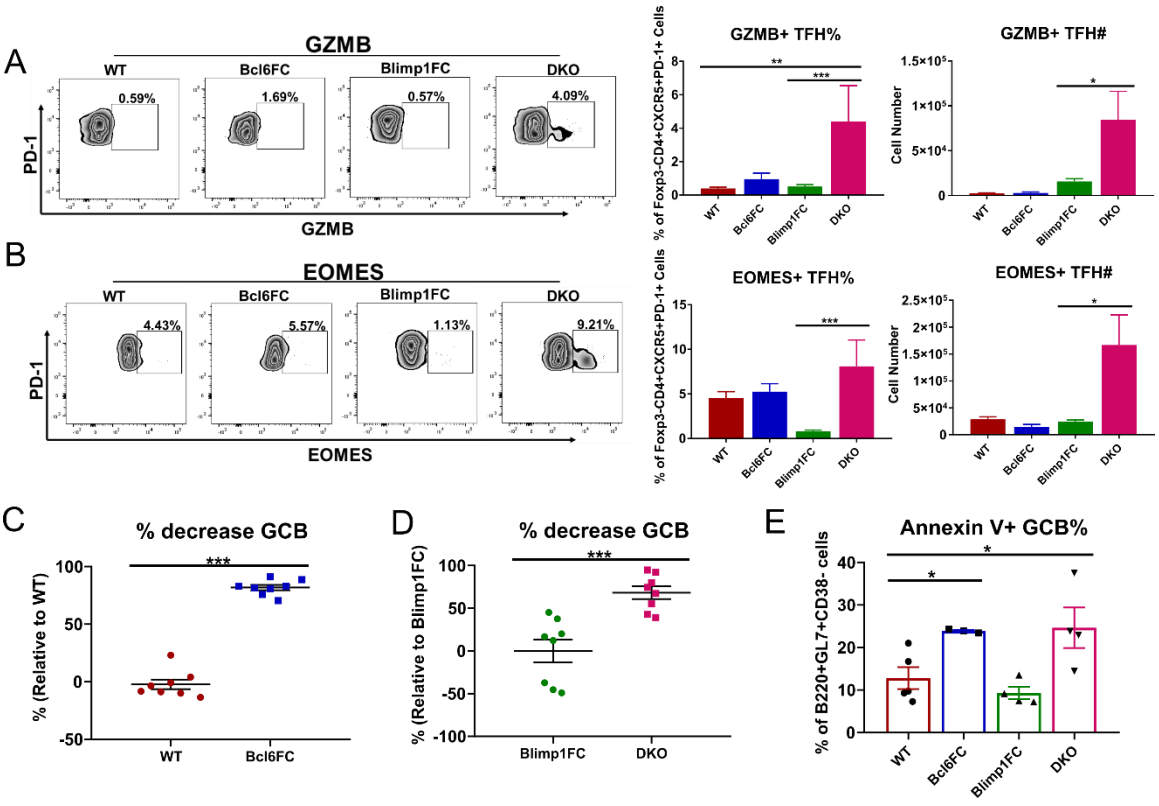

Figure 37: Cytotoxic gene expression in TFH cells and increased GCB apoptosis in TFR-deficient mice. WT, Bcl6FC, Blimp1FC and DKO mice were immunized with SRBC and spleens (SP) were analyzed for the indicated markers and cell populations by flow cytometry. (A-B) Analysis of CD4+FOXP3- PD-1+CXCR5+ TFH cells 9 days after immunization for either staining with (A) anti-granzyme B (Gzmb) or (B) anti-Eomesodermin (Eomes) Ab. Average Gzmb+ and Eomes+ TFH cells are quantitated and graphed as a percentage of TFH cells, and absolute number. N= 4; experiment was repeated 3 times. (C-D) Percent decrease in B220+CD38- GL7+ GC B cells 9 days after immunization in (C) Bcl6FC mice compared to WT mice, and (D) DKO mice compared to Blimp1FC mice. N= 8; experiment was repeated once. (E) Average Annexin V+ GC B cells in WT, Bcl6FC, Blimp1FC and DKO mice, 3 days after immunization. N= 4; experiment was repeated 3 times.

Cytotoxic gene expression in TFH cells and increased GCB apoptosis in TFR-deficient mice

We next set out to characterize Gzmb-expressing TFH cells and their potential role in killing GC B cells. Here we immunized with SRBCs, a model Ag that induces strong TFH responses. We stained TFH cells from WT, Bcl6FC, Blimp1FC and DKO mice for Granzyme B protein by flow cytometry, and saw a slight increase in Granzyme B in Bcl6FC TFH cells (Fig. 37A). However, we found a clearly distinct population of Granzyme B+ cells in the DKO TFH cells, consistent with the higher level of CD8 effector gene up-regulation in these cells (Fig. 37A). We next examined Eomesodermin (Eomes), a protein highly expressed in CD8 effector T cells and cytotoxic CD4 T cells (203). DKO TFH cells had a clear population of Eomes+ TFH cells (Fig. 37B). We were also to detect a similar population of Klrg1+ DKO TFH cells that we could isolate by FACS to test gene expression (Fig. 38A). Klrg1+ DKO TFH cells had dramatically higher Gzmb mRNA and significantly lower expression of the TFH genes Bcl6, Il4 and Il21 (Fig. 38B). Thus, cytotoxic TFH cells may be less effective helper cells and may also actively kill GC B cells. Similar to our results with PCT immunization, we also observed very strong loss of GC B cell response in TFR-deficient mice after SRBC immunization (Fig. 37C, D). These data suggest that cytotoxic TFH cells might be able to kill GC B cells, leading to the loss of GC B cells we see in TFR-deficient mice. Although we were unable to demonstrate cytotoxic activity of TFH cells from DKO mice against GC B cells using *in vitro* assays, we could observe a 2-fold increase in apoptotic GC B cells in Bcl6FC and DKO mice (Fig. 37E). Taken

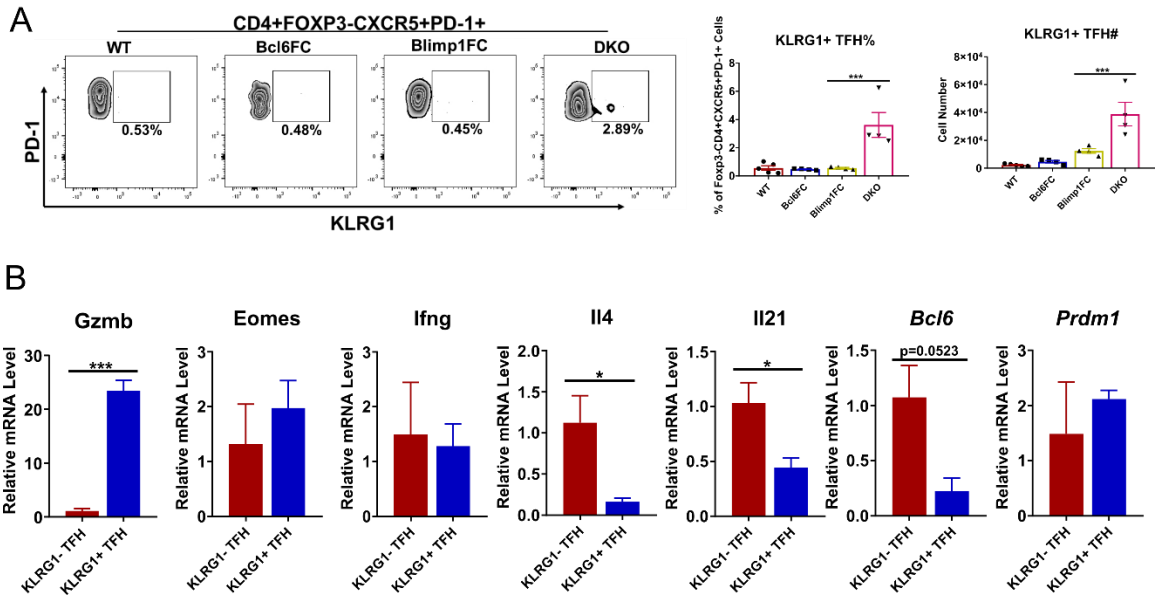

Figure 38: Cytotoxic gene expression is enriched in Klrg1+ TFH cells. WT, Bcl6FC, Blimp1FC and DKO mice were immunized with SRBC and spleens were analyzed for Klrg1 on TFH cell populations by flow cytometry. (A) Analysis of CD4+FOXP3-PD-1+CXCR5+ TFH cells for Klrg1 expression 9 days after immunization. Average Klrg1+ TFH cells are quantitated and graphed as a percentage of TFH cells, and absolute number. N= 4; experiment was repeated 3 times. (B) DKO TFH cells were separated into Klrg1+ and Klrg1- populations by FACS, and RNA was prepared directly after sorting. RT-QPCR was used to analyze gene expression in Klrg1+ and Klrg1- TFH cells. Graphs show relative expression for the indicated genes with the mRNA level in Klrg1- cells set as 1.

together, our data define a new cytotoxic-like TFH cell subset regulated by TFR cells and likely by Stat4. Gzmb-expressing circulating TFH cells have been found in humans, and the development of this population is Stat3-dependent (204). We were unable to find an enrichment of Stat3 target genes in our TFH cell data, suggesting that there can be different origins of Gzmb-expressing TFH cells.

DISCUSSION

T follicular helper cells

TFH cells orchestrate the germinal center reaction and the production of high affinity Abs to Ag, yet the pathways for how TFH cells develop are not well understood, either *in vivo* or *in vitro*. We have uncovered a completely novel mode of inducing the master TFH cell regulator Bcl6, which involves the metabolic sensor AMPK. We also analyzed the critical interplay between two transcription factors that control TFH cell development in opposing pathways: Bcl6 and Blimp1.

Our work ties into extensive recent work showing effector T cell responses are promoted by glycolysis and AMPK (96, 185, 186, 205), however TFH cell responses were not analyzed in these previously published studies. Our findings add a new twist to metabolic regulation of T cells, and indicate that inhibition of glycolysis, with resulting activation of AMPK, induces Bcl6 expression, which can promote TFH cell responses. Although previous studies have shown that Bcl6 is positively regulated by AMPK in endothelial cells (191), and that Bcl6 is up-regulated by glucose deprivation (GD) in pancreatic cancer cells (171), our data here shows that this pathway is operational in CD4 T cells, both in mice and humans, and that this pathway can impact TFH cell differentiation.

Curiously, TFH cells have a unique metabolic profile, with a dampened metabolism compared to other effector T cells (170). This dampened metabolism may be explained in part because Bcl6 is able to repress genes in the glycolysis

pathway (172). Thus, if Bcl6 suppresses glycolysis, this can contribute to the decreased glycolysis in TFH cells. An unresolved question is how this dampened metabolism contributes to the function of TFH cells. In the GC, B cells present cognate Ag to T_{FH} cells and stimulate the TFH cells through the TCR to allow for elaboration of helper cytokines. TFH cells need to be specifically activated in order to help GC B cells, and it has been proposed that strict control of cytokine secretion by TFH cells is a key aspect of their function (109, 206). How cytokine expression is controlled in TFH cells following interaction with GC B cells presenting cognate Ag is a particularly important question for understanding B cell selection in the GC. We propose that metabolism is normally dampened in TFH cells specifically to limit the non-specific secretion of cytokines by TFH cells in the GC, and that metabolism is dramatically activated in TFH cells that are stimulated by cognate GC B cells.

Previous studies have found that Bcl6 is induced by AMPK in endothelial cells (191), and that Bcl6 is up-regulated following glucose deprivation (GD) in pancreatic cancer cells (171), these studies presented different mechanisms for how Bcl6 is induced. In the endothelial cell study, AMPK was shown to inactivate PARP1, which normally represses BCL6 (191), and thus AMPK signaling relieves repression on BCL6. In the GD study, activated Foxo1 was shown to induce Bcl6 expression (171). Our laboratory showed many years ago that Foxo4 positively regulates Bcl6 (207), and AMPK can regulate multiple Foxo factors (208). Interestingly, Foxo1 was shown to repress early TFH cell differentiation, but also was required for maximal Bcl6 expression and full TFH cell differentiation (209).

Overall, it is likely that the regulatory pathway for how AMPK activates BCL6 is complex and involves several factors, but a common theme is factors that respond to metabolic signals. Apart from activation by AMPK, PARP-1 utilizes the metabolic product NAD+ for its enzymatic function, and is thus responsive to the metabolic state of the cell (210).

Although we found Bcl6 was strongly induced when glycolysis was inhibited, inhibition of glycolysis is also detrimental to T cell activation and proliferation (96, 185, 186, 205). Thus, during a strong T cell proliferative response, abundant glucose is required, and it is unlikely that Bcl6 will be activated by AMPK signaling. We hypothesize that during a strong T cell response, glucose can get depleted locally, and this may lead to Bcl6 activation via AMPK. Indeed, we showed that Bcl6 was induced by GD following initial T cell activation in the presence of glucose. Although speculative, this is a plausible pathway for the development of TFH cells during an active immune response, and importantly it is a way for TFH cells to develop in the presence of inhibitory IL-2.

The fact that Bcl6 represses glycolysis (172) makes the activation of Bcl6 transcription by GD, where glycolysis is inhibited, remarkable. This connection indicates that Bcl6 may be part of a feedback loop used by the cell to shut off glycolysis, when glucose is in short supply. This potential regulatory circuit is novel and a subject of future research. Whether induction of Bcl6 by AMPK has different effects on glycolysis in GD conditions versus when glucose is present is another

important issue for future investigation. Ultimately, these findings have therapeutic potential, since targeting AMPK by pharmaceutical agents can be used for augmenting TFH cells during vaccination, or for inhibiting TFH cells in an auto-immune disease setting.

To better understand the pathways for how TFH cells develop, we analyzed the critical interplay between two transcription factors that control TFH cell development in opposing pathways: Bcl6 and Blimp1. Despite the recent characterization of Bcl6 target genes in CD4 T cells (101, 102), the key mechanisms by which Bcl6 promotes TFH cell differentiation are not well understood. A frequently cited pathway proposed for Bcl6-mediated control of TFH cell differentiation is that Bcl6 inhibits terminal CD4 T cell differentiation by repressing the *Prdm1* (Blimp1) transcription (51, 70, 82, 87, 97). Blimp1 is a potent inhibitor of TFH cell differentiation, but the mechanism by which Blimp1 represses TFH cell differentiation has not been clarified. Using mice doubly deficient in both Bcl6 and Blimp1, we demonstrate that Blimp1 does not repress TFH cell differentiation in the absence of Bcl6 function. This data indicates that the major role of Blimp1 in inhibiting TFH cell differentiation is by repressing Bcl6 expression, and this finding clarifies an important aspect of the transcriptional control of TFH cell differentiation.

We also investigated the role of Bcl6 and Blimp1 in controlling TFR cell differentiation. The signaling pathways that control TFR cell differentiation are

poorly understood, but experimental evidence indicates that TFR cells differentiate from both Foxp3+ regulatory T cells that up-regulate Bcl6 as they become TFR cells (106, 211), and also from Foxp3- naive CD4 T cells that up-regulate both Bcl6 and Foxp3 (54). TFR cells require Foxp3, Bcl6 and Stat3 function for their development (50, 106, 109, 110, 211), but other genes that critically control their development have not been well characterized. Here we show that Blimp1 can repress TFR cell differentiation by a Bcl6-independent pathway. One mechanism for this effect may relate to regulation of NFATc1/NFAT2, which has been shown to be important for up-regulation of CXCR5 on TFR cells as well as for expression of PD-1 (105, 118). Blimp1 has been shown to repress NFATc1/NFAT2 expression (105), and thus Blimp1 should have a suppressive role for CXCR5 and PD-1, both of which are key genes increased in TFR cells. Increased expression of NFATc1/NFAT2 in Blimp1-deficient regulatory T cells could lead to Bcl6-independent expression of CXCR5 and PD-1, and appearance of TFR–like cells. Whether the TFR–like cells that develop in dcKO mice are true functional TFR cells is difficult to determine since TFH cells and GCB cells do not develop. We expect that Blimp1-deficient Foxp3+ regulatory T cells with increased CXCR5 and PD-1 would have suppressive function and have access to the B cell follicle. However, these cells may have altered expression of suppressive cytokines, survival and kinetics in the absence of Bcl6. Notably, Blimp1 promotes expression of the important suppressive cytokine IL-10 (192, 212), and thus Blimp1-deficient TFR–like cells likely have decreased IL-10 and may have altered suppressive activity. However, the role of IL-10 in TFR cells is not yet established, and this question

cannot yet be answered definitively. Since TFR–like cells develop in dcKO mice, we cannot completely rule out that TFH cells also develop in dcKO mice but that the TFR–like cells suppress the growth of this population and keep them at an insignificant level. One argument against this idea is our previous work showing that TFR cells do not actually limit the size of the TFH cell population (109, 110).

Our data revealed a new insight into IL-10 regulation, since we showed here that Bcl6 represses transcription of *Il10* independently of Blimp1 in primary CD4 T cells. Since Blimp1 has been shown to promote IL-10 expression (192, 212), one possibility was that Bcl6 represses *Il10* transcription indirectly by repressing *Prdm1* (Blimp1) transcription. Thus our data rules out a mechanism of IL-10 control where Bcl6 acts on IL-10 by repressing Blimp1 and causing decreased *Il10* transcription, and shows that Bcl6 is a direct repressor of *Il10* expression. Indeed, Bcl6 has been shown to bind to a distal enhancer region for both mouse and human *Il10* genes (88, 213), but it has not been clear if this binding leads to functional repression by Bcl6. Our data support the idea that this is a key regulatory region for Bcl6-mediated repression of *Il10.*

Bcl6 has been shown to promote PD-1 expression previously (104), but the mechanism for this regulation has not been defined. This pathway is unusual since Bcl6 is primarily, if not exclusively, a transcriptional repressor protein, and there has long been a question about how Bcl6 can activate genes that are induced at high levels in TFH cells such as PD-1 and CXCR5. One early explanation was

that microRNAs that suppress TFH cell markers are themselves targets of Bcl6, and thus Bcl6 induces TFH cell gene expression by silencing these suppressive microRNAs (193). However, this microRNA pathway has not been supported by subsequent research and more recently microRNAs thought to be suppressed by Bcl6 were shown to be important for TFH cell development (214, 215). We tested the role of microRNAs in induction of PD-1 and CXCR5 by Bcl6 using our RV system, and found that Bcl6 could promote PD-1 and CXCR5 in *Dicer* cKO T cells, which are unable to generate microRNAs. In the present study, we identified a novel Tbet-dependent pathway for the induction of PD-1 by Bcl6. Tbet is a transcriptional repressor of PD-1 gene expression (194), and Bcl6 is a potent negative regulator of Tbet (195). We therefore demonstrated that Bcl6 could counter-repress the negative regulation of PD-1 by Tbet. The unexpected finding was that even though Blimp1 is a potent negative repressor of PD-1, Bcl6-mediated positive regulation of PD-1 acts independently of Bcl6 repressing Blimp1. In summary, we have clarified several aspects of Bcl6 control over TFH cell and TFR cell differentiation. Specifically, we have found that Blimp1 primarily represses TFH cell differentiation through a Bcl6-dependent pathway, Blimp1 represses TFR cell differentiation through both Bcl6-dependent and Bcl6-independent pathways and Bcl6 can promote PD-1 expression by a novel Blimp1-independent mechanism involving Tbet inhibition.

T follicular regulatory cells

T follicular regulatory cells help control the Germinal Center (GC) and Ab response and the function of TFR cells in the GC is not well understood, however the dominant paradigm in the TFR field has been that TFR cells repress excessive TFH and GC B cell proliferation and help promote stringent selection of high affinity B cells. Newer mouse models have allowed the study of TFR cell function with more precision than previous approaches. Here we show for the first time that Ag-specific IgE responses induced in a food allergy challenge system are uniquely dependent on TFR cells and that TFR cells play an active helper role in the production of IgE. Our data also point to a key role of IL-10 made by TFR cells in promoting the development of IgE secreting cells from the GC. The data presented here fit into an emerging helper model of TFR cell function that reshapes the standard view of TFR cells.

The first studies of TFR cells described them as suppressors of the GC and Ab response (49, 50, 106), and most studies on TFR cells have operated within this conceptual framework of TFR cells as suppressors (111, 216). Recently however, a different model has emerged-- that TFR cells act as helper cells within the GC to facilitate GCB cell maintenance and Ag-specific Ab production. Laidlaw *et al* showed a key helper function for TFR cells in the GC was producing IL-10 that helped promote GCB cell entry into the dark zone and maintaining the size of the GC (68). Laidlaw *et al* studied TFR function after Lymphocytic Chorio-Meningitis Virus (LCMV) infection (68), a very different type of immune response

than the food allergy model studied here. While Laidlaw *et al* observed significant decreases in GC size from loss of TFR cell-derived IL-10, comparable to what we observed in our study here, the effects on virus-specific IgG were modest (68). Our earlier work with TFR-deficient mice also showed a relatively small loss of Ag-specific IgG using model Ags such as Sheep Red Blood Cells (110). In the original studies that reported TFR cells, mixed bone marrow methods were used to look at the functions of TFR cells (49, 50). A few other studies also used *in vitro* co-culture system to study TFR cells which showed some interesting findings (149, 217). In our lab, a TFR-deficient mouse model where the *Bcl6* gene is specifically deleted in Foxp3+ T cells (*Bcl6*-flox/flox Foxp3-Cre or Bcl6FC mice) has been used by us and others to study TFR cell function in immune and autoimmune responses. We think this Bcl6FC mouse model is a more accurate tool for the study TFR cells though knockout *Bcl6* gene in Tregs may have some other unexpected cell intrinsic effects. Here we show a complete loss of Ag-specific IgE in the absence of TFR cells, indicating that there is a particularly critical role of TFR cells to act as helpers in the allergic IgE response. Our data also show that blockade of IL-10 signaling on B cells mimics the effect of loss of TFR cells specifically on Ag-specific IgE, indicating that TFR cell-derived IL-10 is likely to mediate the positive effect of TFR cells in this response.

One major question from our data is therefore why GC-dependent IgE responses appear to be much more sensitive to the loss of TFR cells than GC-dependent IgG responses. The answer is likely to lie with the unique nature of IgE

as an Ag receptor for B cells in the GC. Specifically, IgE+ switched GC B cells have altered Ag receptor signaling, are unstable and display increased apoptosis, and selection of IgE+ B cells in the GC is less efficient than with B cells expressing other Ig isotypes (218-222). Interestingly, inhibition of apoptosis in the GC helps to promote IgE responses (223). A previous study showed that IgE+ GC B cells have little to do with generating the high-affinity memory IgE response (224). Recently, both mouse and human studies showed that high-affinity IgE producing plasma B cells are generated from IgG-memory B cells upon re-exposure to Ag through additional class-switch recombination to IgE (225, 226). The precursors of IgE+ plasmablasts appear to be IgG1+ memory B cells in mice and IgG4+ memory B cells in humans (225, 226). These IgG+ memory B cells come from a switch from IgM to IgG and are thought to be generated in the GC (225-227). We have found that IgG1+ GC B cells were significantly decreased in our Bcl6FC mice after PCT challenge on D36 compared with WT (data not shown). It is highly possible that TFR cells positively promote the switch of IgG1+ memory B cells into high-affinity IgE producing plasmablasts, though more work is needed to address this. Similarly, IL-10 may play a fundamental role in the regulation of this IgE switching and it may directly affect the generation of IgG1+ memory B cells within the GC for we showed decreased GC B cells in MB1-Il10ra-/- mice. We propose that IL-10 made by TFR cells helps to stabilize GC B cells and promotes the generation of stable high-affinity IgE-secreting plasmablasts. Thus, for allergic diseases mediated by IgE, TFR cells represent an important new target of control over IgE production.

Our data have significant implications for food allergy responses and other IgE-mediated allergic diseases. Since IgE is a central mediator of allergic disease, much research has gone into developing inhibitors of IgE and IgE-induced responses (228-231). Our data shows that blocking IL-10 signaling after the initiation of the peanut allergy challenge can potently inhibit the production of peanut-specific IgE. This suggests that IL-10 is a new mediator of IgE responses and may be exploited to inhibit IgE-mediated allergy. IL-10 is a multi-functional cytokine with both positive and negative regulatory function and is classically considered a key suppressive cytokine. Recent results suggest that IL-10 produced by TFR cells is also a major positive factor for Ab responses that may be a good target for inhibition in the right type of allergic disease, such as food allergy.

IL-10 is becoming more widely seen as a positive factor in GCB cell responses (68, 139, 232), but our study is the first to show the regulation of IgE+ GCB cell responses by IL-10. More research is required to determine whether IL-10 non-specifically promotes the survival of IgE+ GC B cell responses after the switching stage or whether IL-10 might specifically increase the early induction of IgE switching. Additionally, more studies are needed to understand if TFR cells can act both as suppressors and helpers depending on the exact type of immune response, or if TFR cells default towards a helper role for immune responses to non-self Ags (216). Despite these questions, our work has opened a new

understanding of the regulation of IgE responses as well as helped us recognize the complex function of TFR cells in the GC.

Our data in the study of cytotoxic TFH cells define a new cytotoxic-like TFH cell subset that is repressed by TFR cells and is promoted by pro-inflammatory signals (e.g. virus infection or Treg cell defects). These Gzmb-expressing TFH cells have the potential to kill B cells in the GC. Our findings therefore reveal a new regulatory pathway for the GC and high-affinity Ab production.

TFR cells have been generally considered to repress the GC and Ab response (111, 216, 233, 234). Our data here show a very clear and potent helper effect of TFR cells in the GC, in two different types of immune challenge (PCT and SRBC). Thus our data is more consistent with a recent study that showed that TFR cells can help promote the Ag-specific IgG and GC response by expressing IL-10 (68). However, our data reveal a new pathway for TFR cells to help the GC response, by inhibiting the development of cytotoxic-like TFH cells.

Our data indicate that the aberrant TFH cell phenotype that develops in the absence of TFR cells can also develop in a TFR cell sufficient GC response after virus infection (Fig. 36C). Gzmb-expressing circulating TFH cells have been found in humans, and the development of this population is Stat3-dependent (204). However, we were unable to find an enrichment of Stat3 target genes in our TFH cell data (not shown), suggesting there may be different origins for these circulating

Gzmb-expressing TFH cells. We saw a strong correlation of TFH cytotoxic genes with Stat4 regulation. This may indicate that IL-12 is increased in the GC environment in the absence of TFR cells. A key function of Tregs is to down-modulate DC function, including the secretion of cytokines such as IL-12 (235, 236). A similar suppression of cytokine secretion by DCs, may also be a mode of TFR cell function in the GC or in the surrounding lymphoid environment.

Very recently, Gzmb-expressing TFH cells with a genotype related to our cytotoxic TFH cells have been found in GCs in human tonsillitis (237). How these human cytotoxic TFH cells develop is not clear but they appear to be a defective or exhausted state of TFH cell differentiation induced by chronic infection (237). It is tempting to speculate that defective TFR function leads to these human Gzmb-expressing TFH cells, but more research is required for this question. However, our results do show the conservation of this cytotoxic TFH cell pathway in humans.

Overall, our findings raise the possibility that cytotoxic TFH cells are a normal part of the GC response and that Gzmb+ TFH cells kill GC B cells and help to fine-tune the Ab response. Furthermore, cytotoxic TFH cells may also play a role in controlling virus infection in the GC. Thus, increased development of these aberrant TFH cells may be beneficial after virus infection. The precise function of TFR cells in suppressing Gzmb-expressing TFH cells will be an important area for future research.

FUTURE DIRECTIONS

Here we documented that Bcl6 is strongly induced when glycolysis is inhibited, the fact that Bcl6 represses glycolysis (172) makes the activation of Bcl6 transcription by GD, where glycolysis is inhibited, remarkable. This connection indicates that Bcl6 may be part of an important feedback loop used by the cell to shut off glycolysis, when glucose is in short supply. This potential regulatory circuit is very novel and a subject of future research. Whether induction of Bcl6 by AMPK has different effects on glycolysis in GD conditions versus when glucose is present is another important issue for future investigation. These findings have therapeutic potential, since targeting AMPK by pharmaceutical agents can be used for augmenting TFH cells during vaccination, or for inhibiting TFH cells in an auto-immune disease setting.

Many transcription factors may be involved in the induction of Bcl6 expression by GD such as the deacteylase Sirtuin-1 (Sirt1) which can be activated by AMPK (238, 239). We could use primary mouse naïve CD4+ T cells in secondary stimulation cultures and test compounds such as EX527, the highly specific inhibitor for Sirt1 (84, 239). Additionally, Bcl6 self-repression is uniquely dependent upon the co-repressor protein CTBP, loss of CTBP binding, may also converge on the Bcl6 promoter to increase transcription. The binding of Bcl6 and CTBP factors to the Bcl6 promoter and other regulatory regions of the Bcl6 gene has already been established in multiple studies (84). We may use EL4 cells for Chromatin immunoprecipitation (CHIP) assays initially, with Abs to each of these

factors. Bcl6 and CTBP are expected to be bound at a higher rate to the *Bcl6* gene in +glucose conditions. We may also use the inhibitor MTOB (4-methylthio-2-oxobutanoate) in our study to look at Bcl6 expression under different conditions (240).

Though TFH cells have been studied extensively, TFR cells have only been analyzed in a very small fraction of infectious disease models and immunological diseases such as allergy and autoimmunity. Testing TFR cell function in various disease states will be an important area for future research on TFR cells. Also unknown is if TFR cells affect diseases that are not driven by Ab-mediated pathology. Whether TFR cells play a regulatory role in cancer, diabetes, heart disease, atherosclerosis, or other types of inflammatory diseases, is open for exploration. The mechanism of TFR cell help in the GC is not completely understood and an important topic is why some types of GC responses seem to rely on TFR cells for help whereas other GC responses are only mildly affected or not affected at all by loss of TFR cells. A major question for future studies is whether TFR cells switch between help and suppression in the GC for foreign Ag, or primarily act as helpers for foreign Ag and suppressors of autoimmune responses. If TFR cells act as suppressor cells of non-autoimmune responses in the GC, what mechanism of suppression do they use, and what controls whether TFR cells act as suppressors versus helpers? Do human and mouse TFR cells have similar helper and repressor functions? TFR-like cells have been found circulating in both mice and humans; what is the relationship of these cells to TFR

cells in the GC? Also unclear is how TFR cells regulate Ab affinity maturation and TFH responses. A major question is whether TFR cells regulate the generation or differentiation or survival of memory B cells. Lastly, almost nothing is known about what signals drive TFR cell responses to the GC and what Ags do they recognize? Thus, there are huge numbers of vital questions about TFR cells that need to be answered through more research.

In our food allergy model, we still don't know if TFR cells directly affect IgE+ plasmablast cell differentiation. An effect of increased survival or proliferation on IgE+ GC B cells may be difficult to detect if these cells differentiate rapidly into IgE+ plasmablasts and plasma cells. Nonetheless, further study is needed to analyze GCB cell and plasma cell responses. We could use flow cytometry to assess GC B cells that have switched to IgG1 or IgE at each time-point in each mouse model. This part of data would provide some preliminary information regarding IgE+ Ab producing cell regulation. PtenFC mice may be used as a positive model here for this study purpose. Little is known how IL-10 regulates IgE responses, an obvious question is does IL-10 promote IgE+ GCB proliferation or promote Ab isotype switching to IgG1 then IgE? *Il10ra* cKO mice which have defect of IL-10 signaling in B cells may be used with IgE reporter mice to track IgE+ B cell development.

For the cytotoxic TFH cell pathway, more functional studies of these TFH cells need to be carried out. Either Gzmb inhibitor or *Gzmb* gene deficiency may

be applied to our DKO mice to test the function of TFH cell cytotoxicity. Is there any change of GCB cells when *Gzmb* is knocked out in T cells? It will also be interesting to see if humoral Ab responses are affected by Gzmb inhibition or not. In our data presented above we showed that cytotoxic TFH cells seem to be STAT4 dependent, we have some preliminary data showing that Il12a expression is highly upregulated in DCs of DKO mice (data not shown), but how this upregulated IL-12/STAT4 signaling impacts TFH phenotype remains unknown. *In vivo* blocking of IL-12 of DKO mice using blocking Ab may partially address if IL-12 is fundamental for the development of cytotoxic TFH cells.

Even though Tregs act overwhelmingly as major suppressors of the immune response, TFR cells provide a striking and clear example of Treg cells acting as “helper” cells for the immune response. At least part of this TFR cell helper function is producing IL-10 that promotes GC B cell growth and the GC-dependent high affinity Ab response. Thus, in the context of the GC response, TFR cells appear to maintain a key balance between help (GC maintenance, Ab response, Ab affinity) and suppression (TFH cell numbers, GC B cell numbers, TFH cell cytokines, auto-Abs). One interesting idea is that the auto-reactivity and suppressive capability of Treg is used to help control auto-immunity in the GC but has been co-opted to also promote the overall GC response. Additional future work is needed to fully understand the role of TFR cells in the overall humoral immune response, and in the larger scope of the immune system.

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INVITED TALKS:

“Surprising roles for T regulatory cells in antibody regulation” Department of Microbiology, Southern Illinois University, Carbondale, IL. 09-28-2018

ABSTRACTS OF CONFERENCES:

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